Effect of cholesterol and cholesterol analogues on Platelet Function

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This thesis has been composed by myself and all the experimental results described herein, except where otherwise acknowledged, are the product of my own work.

Signed:

To Jane

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Abstract

This thesis reports studies carried out to investigate further the effect of alteration of the platelet cholesterol content on platelet function. Recent research has shown that cholesterol-enriched human platelets were capable of synthesising greater quantities of pro-aggregating compounds compared to control and cholesterol-depleted platelets. Phospholipase A_2 activity in the platelet membrane may be an important rate limiting step in the metabolism of arachidonic acid to pro-aggregating compounds for example thromboxane A_2. Thus in the present project, cholesterol-enriched platelets have been investigated with particular reference to phospholipase A_2 activity.

Rat platelets were enriched with and depleted of cholesterol by *in vitro* incubation with cholesterol-rich and cholesterol-poor phospholipid liposome suspensions respectively. Aggregation studies showed that cholesterol-enriched rat platelets (in platelet-liposome mixtures) were more sensitive to collagen and ADP induced aggregation. Phospholipase A_2 assays were carried out by analysing the conversion of phosphatidylcholine containing a radiolabelled sn-2 fatty acid. Rat platelets were resuspended once, and no difference in phospholipase A_2 activity was detected between cholesterol-enriched and cholesterol-normal platelets.

Rabbits were fed a cholesterol-rich diet for 4 weeks. Platelets isolated from cholesterol fed rabbits had a significantly higher cholesterol:phospholipid molar ratio than platelets from rabbits fed a normal diet. The once resuspended cholesterol-enriched rabbit platelets showed significantly higher phospholipase A_2 activity compared to control platelets treated in the same way. Also, the cholesterol-enriched rabbit platelets were observed to have a more

active arachidonic acid metabolic pathway, as assayed by measuring arachidonic acid induced thromboxane A_2 and MDA production.

Platelets were also examined from hypercholesterolaemic human subjects and compared with platelets from normal human subjects. In these experiments, no differences were detected in cholesterol content or phospholipase A_2 activities of crude platelet membrane fractions prepared from these platelets. This may have been due to this assay not being suitable for the very labile human platelet phospholipase A_2 activity.

Crude platelet membrane fractions were also prepared from rat platelets with altered cholesterol content. Phospholipase A_2 assays consistently showed that the activity was significantly higher in cholesterol-enriched rat platelet membrane fractions than in control platelet membrane fractions. These results and those from the rabbit platelet experiments suggested that platelet hypersensitivity induced by cholesterol-enrichment may be mediated partly through hyperactivity of the membrane bound phospholipase A_2.

Rat platelets were also enriched with cholesterol analogues which were different from cholesterol only in that they had side chains of reduced length. The analogues studied were pregn-5-en-3β-ol, chol-5-en-3β-ol, and 27 norcholest-5-en-3β-ol. These analogues were readily taken up by the platelets on incubation with analogue loaded liposome suspensions. Aggregation tests and scanning electron microscopy indicated that major changes occurred in the physiology of platelets enriched with analogues which had a side chain 3 or more carbon atoms shorter than cholesterol. Enrichment of rat platelets with the analogue which had a side chain shorter by one carbon atom had the same effects as cholesterol-enrichment. These platelets became hypersensitive to collagen induced aggregation.

The results presented in this thesis suggest that the activity of phospholipase A_2 may be influenced by the membrane cholesterol content, and clearly indicate a requirement by the platelet membrane of a sterol of precise dimensions to maintain normal platelet function.

Abbreviations

C_{21}-analogue, C_{21}-sterol	pregn-5-en-3β-ol
C_{24}-analogue, C_{24}-sterol	chol-5-en-3β-ol
C_{24}-analogue, C_{26}-sterol	27 norcholest-5-en-3β-ol
cholesterol	cholest-5-en-3β-ol
ADP	adenosine 5'-diphosphate
cAMP	adenosine 3,5'-monophosphate
ATP	adenosine 5'-triphosphate
HHT	12 L-hydroxy-5,8,10-heptadecatrienoic acid
HETE	12 L-hydroxy-5,8,10,14-eicosatetraenoic acid
HPETE	12 L-hydroperoxy-5,8,10,14-eicosatetraenoic acid
PG	prostaglandin
MDA	malondialdehyde
HMG CoA	3-hydroxy-3-methylglutaryl-coenzyme A
LDL	low density lipoprotein
EDTA	ethylenediamine tetra-acetate (disodium salt)
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
DPPC	L-α dipalmitoyl phosphatidylcholine
Lecithin, PC	phosphatidylcholine
[1-^{14}C]oleoyl PC	1-acyl-2-[1-^{14}C]oleoyl phosphatidylcholine
[1-^{14}C]arachidonyl PC	1-acyl-2-[1-^{14}C]arachidonyl phosphatidylcholine
SR	sarcoplasmic reticulum
tlc	thin layer chromatography
PRP	platelet rich plasma
PPP	platelet poor plasma
PI	phosphatidylinositol
DMSO	dimethylsulphoxide
FFA albumin	fatty acid free albumin

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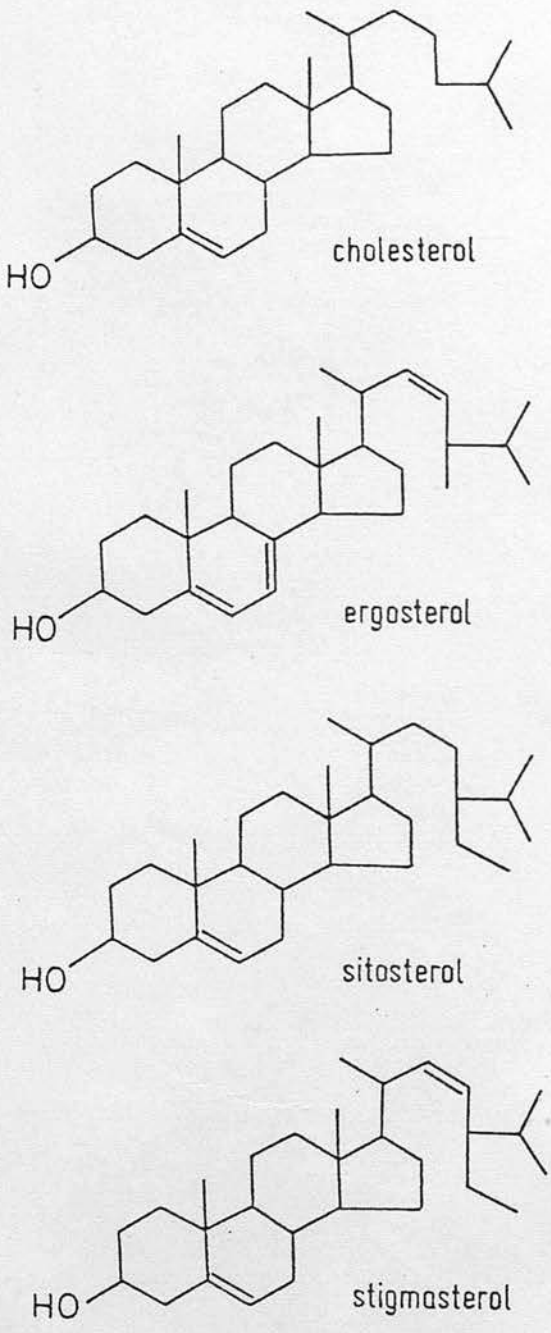
Chapter 1

Introduction

Figure 1.1. Structures of natural sterols



1.1 Outline of Introduction

There are various areas of research which are of interest to the studies carried out and presented in this thesis. The introductory chapter has been divided into sections in order to review the different fields of research with clarity and emphasis.

The first section reviews briefly some of the research carried out on artificial membrane systems to investigate the effect of cholesterol. The second section discusses some examples of studies carried out on more complex biological (i.e. natural) membranes. In this section, studies performed to investigate the effects of cholesterol on red blood cells are discussed as these studies preceeded research into the effects of cholesterol on platelets. This latter research is discussed later in the chapter (see Section 1.6) after the sections discussing platelet function (1.4) and platelet arachidonic acid metabolism (1.5). In Section 1.6, the research which has been carried out into the effect of alteration of platelet cholesterol content is discussed. In this section, research carried out on platelets from subjects with hypercholesterolaemia is also reviewed. The relevance of the research project presented in this thesis to clinical studies is suggested. The final section briefly outlines the strategy of this project.

1.2 Distribution of Cholesterol and its effect on Artificial Membranes

Cholesterol is an important component of mammalian cell membranes. It is not present in bacterial cells, but related sterols are present in plants and fungi of yeast. These sterols possess a double bond in the side chain at the 22nd carbon atom and/or a methyl or ethyl group at the 24th carbon atom (see Figure 1.1). Stigmasterol and sitosterol are found in plants, and ergosterol is found in fungi and yeast.

The quantity of cholesterol found in mammalian cell membranes varies depending on the source. Some membranes contain high levels of

cholesterol, for example liver cell plasma membranes have been reported to contain a cholesterol to phospholipid molar ratio of 0.83 (Dorling and Le Page, 1973). The ratio in erythrocyte membranes was also high at 0.90 while the ratio in subcellular membranes, nuclei, mitochondria and microsomes from liver, ranged from 0.11 to 0.33 (Ashworth and Green, 1966). Cholesterol is therefore a major component of cell membranes, and research into its role in membranes has received much attention.

Initial studies concentrated on examining the role of cholesterol in artificial membrane systems, since biological membranes are highly complex. In such artificial systems, the interaction of cholesterol with membrane components, particularly phospholipid, has been investigated. The model systems developed have included monolayers and liposomes consisting of phospholipid and various quantities of cholesterol. Several physical techniques have been used in these studies including electron paramagnetic resonance (epr), nuclear magnetic resonance (nmr), spectroscopy, X-ray diffraction, and differential scanning calorimetry (for review see Demel and De Kruyff, 1976).

The overall effect of incorporation of cholesterol is considered to be the production of a state of intermediate fluidity in membranes between a crystalline or liquid lipid matrix as reviewed by Oldfield and Chapman (1972). In order to determine more precisely the interaction of cholesterol with phospholipids, Hubbell and McConnell (1971) studied the molecular motion of spin labelled phospholipids in phospholipid bilayers containing cholesterol. This spin label was attached at different points along the hydrocarbon fatty acyl chain, and epr spectroscopy showed that a fluidity gradient existed in the bilayer. The central hydrophobic core was fluid in comparison to the

polar region at the surface of the bilayer which was more rigid. The fluidity gradient may arise in a bilayer because the overall motion of the acyl chain is composed of rotations about the individual carbon-carbon bonds. These rotations are transitions between trans and cis conformations. If it is assumed that the fatty acyl chains of the phospholipid molecule are anchored at the polar interface of the bilayer, the chances of trans configuration of a carbon-carbon bond increases with the length of the hydrocarbon chain with a consequent increase in membrane fluidity.

Bruckdorfer et al. (1968) showed that the sterol nucleus structure was critical for interaction of sterol with phospholipid. These workers showed that cholesterol was incorporated into phosphatidylcholine dispersions much more readily than similar sterols studied, which suggested that cholesterol interacted most favourably with the phospholipid. Demel et al. (1972) showed that incorporation into liposomes of 3β-hydroxysterols such as cholesterol, lathosterol, 7-dehydrocholesterol and β-norcholesterol caused the most pronounced reduction in permeability to glucose, glycerol and Rb^+. These workers showed that the 3α-hydroxycholesterols, epicholesterol and androstan-3α-ol had no effect on permeability of these materials. In the same study, neither coprostanol (which has a non-planar sterol nucleus) nor androstan-3β-ol (which has no side chain at the 17th carbon atom) had any effect on permeability.

A structural analysis of egg lecithin-cholesterol bilayers was carried out by Franks (1976) using X-ray diffraction, and by Worcester and Franks (1976) using neutron diffraction. By interpretation of electron density profiles derived from bilayers, Franks (1976) concluded that the hydroxyl group at the 3rd carbon atom was positioned near the water interface. The phospholipid head group was suggested

to lie in the bilayer plane, and the results implied that the hydroxyl group of the cholesterol molecule was positioned adjacent to the sn-2 carbonyl oxygen. Schwartz and Paltauf (1977) showed that hydrogen bonding existed between the hydroxyl group of the cholesterol molecule and the sn-2 carbonyl oxygen of the fatty acid molecule. These workers compared the passive diffusion of Na^+, Cl^-, and glucose across the membrane of single walled liposomes formed from cholesterol and diether phosphatidylcholine (which contained no ester carbonyl group), 1-ether-2-ester phosphatidylcholine and diester phosphatidylcholine. Replacement of the ester groups by the ether groups which cannot hydrogen bond removed the cholesterol mediated reduction in glucose and Na^+ permeability. The hydrogen bonding can only take place when the sterol contains an equatorial hydroxyl group (3β). An axial hydroxyl group (3α) cannot hydrogen bond with the sn-2 carbonyl oxygen because the O-H-O bond angle is not consistent with hydrogen bond formation.

However, more recently Clejan et al. (1979) investigated further the hydrogen bond between the 3β-hydroxyl group of cholesterol and the carbonyl oxygen at the 2 position of PC. These workers suggested that similarities between the effects of sterols on the initial rates and reflection coefficients of polar nonelectrolyte diffusion in diester-PC bilayers and those from phospholipids lacking acyl groups indicated that carbonyl oxygens of the fatty acyl groups in the 1 and 2 positions of PC are not essential to obtain interaction with cholesterol. These results are therefore not in agreement with the results of Schwartz and Paltauf (1977) discussed above (see review by Szoka and Papahadjopoulos, 1980).

The results from the studies discussed above suggested that permeability of sterol-phospholipid liposomes depends on certain

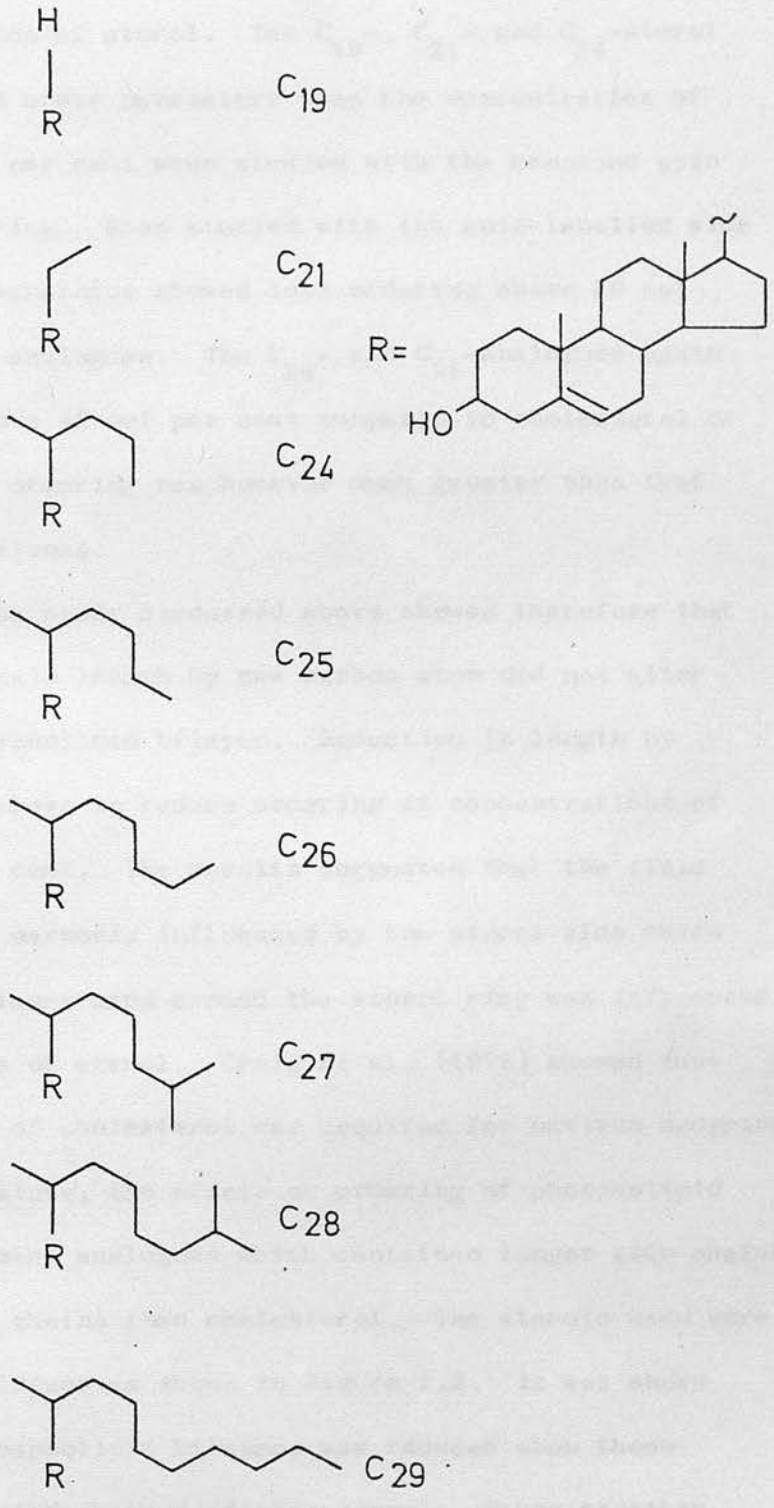
structural requirements of the sterol molecule. These are 1) a planar sterol nucleus 2) possibly a 3β-hydroxyl group although there is some dispute about this and 3) an intact side chain at C_{17}.

The importance of the side chain (indicated above) in the cholesterol molecule has been investigated. Edwards and Green (1972) showed that the incorporation of sterols into liposomes depended on the structure of the side chain. Campesterol (with an additional methyl group branch in the side chain at the 24th carbon atom), and sitosterol (an additional ethyl group at C_{24}) were compared with cholesterol for incorporation into phospholipid liposomes. It was shown that the ability of these sterols to enter liposomes decreased in the order cholesterol, campesterol and sitosterol.

It was shown by Stevens and Green (1972) that the length and degree of branching of the ester of the testosterone molecule influenced its incorporation into phosphatidylcholine liposomes. Maximum incorporation was obtained with testosterone octanoate which has an unbranched eight carbon side chain. The side chain of cholesterol has eight carbon atoms but because of branching in two places the total length is six carbons. Thus the value of the data from the study of Stevens and Green (1972) is questionable because the testosterone octanoate molecule would be considerably longer than cholesterol, and testosterone esters are not closely analogous to cholesterol in their molecular shape and polarity.

Suckling and Boyd (1976) carried out a spin label study to investigate the interactions of the cholesterol side chain with egg phosphatidylcholine liposomes. Analogues of cholesterol were synthesized which retained the cholest-5-en-3β-ol ring system but which had side chains of varying lengths (see Figure 1.2). The effect of sterols on the order parameters of two spin labels in liposomes were examined with proportions of sterol to phospholipid from 0 to 50 mol per cent.

Figure 1.2. Structures of cholesterol analogues synthesised by Craig (1978) and Suckling and Boyd (1976)



The spin labels used were sterols either with the spin label rigidly fixed in the ring system, or with the label incorporated into the side chain. The results showed that the order parameters in liposomes containing cholesterol or the C_{26}-sterol analogue were very similar and increased with increasing proportion of sterol. The C_{19}-, C_{21}- and C_{24}-sterol analogues showed reduced order parameters when the concentration of sterol was above 40 mol per cent when studied with the compound spin labelled in the sterol ring. When studied with the spin-labelled side chain compound, the C_{19}-analogue showed less ordering above 20 mol per cent than the other analogues. The C_{24}- and C_{21}-analogues again showed less ordering above 40 mol per cent compared to cholesterol or the C_{26}-analogue. This ordering was however much greater than that observed for the C_{19}-analogue.

The results from the study discussed above showed therefore that reduction in the side chain length by one carbon atom did not alter the ordering of the phospholipid bilayer. Reduction in length by three carbon atoms was shown to reduce ordering at concentrations of sterol above 40 mol per cent. The results suggested that the fluid core of the bilayer was markedly influenced by the sterol side chain length, and that the bilayer area around the sterol ring was influenced at higher concentrations of sterol. Craig et al. (1978) showed that the complete side chain of cholesterol was required for maximum ordering in liposomes. In this study, the effect on ordering of phospholipid bilayers was examined using analogues which contained longer side chains as well as shorter side chains than cholesterol. The sterols used were C_{25}-, C_{28}-, and C_{29}-analogues as shown in Figure 1.2. It was shown that the ordering of phospholipid bilayers was reduced when these analogues were incorporated instead of cholesterol. Taken together the results obtained with these analogues suggested that the complete

side chain of cholesterol has a specific role to play in the interaction with phospholipid. The cholesterol molecule must be the optimum size to fit into the bilayer to give the maximum possible interaction with the phospholipid.

In a further investigation, Suckling et al. (1979) showed that cholesterol incorporation caused the greatest rigidifying effect in a phospholipid bilayer compared to the sterol analogues and this was shown by several methods. For example, one method showed that the rate of osmotic shrinking of phosphatidylcholine liposomes was substantially reduced with cholesterol incorporation compared to that observed with any of the sterol analogues. Properties of phospholipid monolayers were also examined in this study. Cholesterol was observed to cause substantial condensation of the monolayer when surface pressure-area measurements of the spread films were made. This effect was observed with all the analogues, although the effect was reduced when C_{19}- or C_{21}- analogues were incorporated into the monolayer. These findings suggested that a side chain of moderate length was required and that the ring structure alone was not sufficient for structural stability. In monolayers, the C_{28}-, and C_{29}- analogues behaved no differently from cholesterol. This suggested that the extra length of side chain was permitted to extend freely in space above the subphase. In bilayers however, the cholesterol molecule fitted precisely.

1.3 Effect of Cholesterol in Biological Membranes

Biological membranes have been shown to contain varying quantities of cholesterol depending on the source as discussed above. Since it had been revealed that cholesterol incorporation had a profound effect on the physical properties of phospholipid bilayers, research into the effect of cholesterol in biological membranes has been extensive in recent years. Klappauf and Schubert (1977) for example demonstrated

that Band 3 protein from human erythrocyte membranes interacted strongly with cholesterol. In this study, the protein was extracted without the use of detergents. The Band 3 protein was injected underneath monolayers of different phospholipids (representing the main phospholipid classes of the erythrocyte membrane) and of cholesterol and a pronounced increase in monolayer surface pressure was observed. Thus the effect of cholesterol was studied on a purified natural component. The pressure and rate of change of pressure were much larger for monolayers of cholesterol than phospholipid, over the whole range of protein concentration used. This indicated a stronger interaction of Band 3 protein with cholesterol than with phospholipid.

The effect of cholesterol on membrane components has also been investigated in intact membranes. It was reported that an increase in the proportion of cholesterol to phospholipid in sarcoplasmic reticulum (SR) from rabbit hind leg muscle caused a decrease in the activity of the Ca^{2+}-ATPase (Madden et al., 1979). The activity returned to normal when excess cholesterol was removed from the SR preparation. Cholesterol was delivered and removed from SR by incubating with cholesterol-rich and cholesterol-poor liposomes. The workers suggested that cholesterol interacted with the protein in some way to modulate its activity. Warren et al. (1975) had however previously reported that cholesterol was excluded from the phospholipid annulus surrounding the Ca^{2+}-ATPase from the same source. This annulus consisted of 30 phospholipid molecules (Hesketh et al., 1976) which normally excluded cholesterol and buffered the protein against bulk membrane perturbations.
The results of Madden et al. (1979) and Hesketh et al. (1976) therefore disagree on the interaction of cholesterol with this membrane protein. It is possible that the differences in the preparations were responsible for the different results. Madden et al. (1979) altered the cholesterol

content of native SR membranes with cholesterol rich phospholipid dispersions. Warren et al. (1975) and Hesketh et al. (1976) purified the enzyme and reconstituted the preparation with specific phospholipids with or without cholesterol.

Recently Johannsson et al. (1981) have suggested that the inhibition of the Ca^{2+}-ATPase seen by Madden et al. (1979) by cholesterol enrichment is explained by the spontaneous uncoupling of thermally unstable SR. Johannsson et al. (1981) showed that when dithiothreitol was present in the incubation medium, no inhibition by cholesterol could be seen. However Madden et al. (1981) reinvestigated the problem, and prepared Ca^{2+}-ATPase by the method of Warren et al. (1975), and studied the activity in the presence and absence of dithiothreitol. They observed that in the presence of dithiothreitol, and on addition of A23187, a calcium ionophore, cholesterol enriched SR vesicles showed a smaller increase in Ca^{2+}-ATPase activity than did control SR vesicles. These workers could not say whether the effect of cholesterol was due to either a change in bulk fluidity or a direct effect on the hydrophobic region of the protein. They suggested that because A23187 completely collapsed the Ca^{2+} gradient, a cholesterol induced alteration of membrane fluidity would not have caused the observed effect. They suggested that collapsing the Ca^{2+} gradient in coupled preparations of SR with a Ca^{2+} ionophore allowed expression of the maximum catalytic activity and unmasked the inhibitory effect of cholesterol on the Ca^{2+}-ATPase. Thus cholesterol was demonstrated to influence this membrane protein in a manner which remains unclear.

Recently, further investigations of the influence of cholesterol on erythrocyte membrane proteins have been carried out. Borochov et al. (1979) enriched and depleted erythrocyte membranes of cholesterol and the effects on membrane proteins were assessed using a membrane-

impermeant sulfhydryl reagent. Reaction of this probe with intact cells enabled quantification of outward facing sulfhydryl groups and reaction with leaky ghost membranes permitted quantification of inward facing sulfhydryl groups. They showed that cholesterol enrichment enhanced the surface labelling of Bands 1, 2, 3, and 5, decreased the labelling of Band 6 and did not alter significantly that of Band 4. Thus changes in erythrocyte membrane cholesterol altered the availability of sulfhydryl groups at the membrane surface. Nigg and Cherry (1979) investigated the influence of cholesterol on the rotational diffusion of Band 3 in human erythrocyte membranes. These workers observed flash-induced dichroism of an eosin probe in membranes of various cholesterol content. They showed no significant changes in the rotational diffusion of the protein following variation of membrane cholesterol:phospholipid molar ratios over the range 0.34-1.66. In view of the results obtained by Borochov et al. (1979) that the number of Band 3 sulfhydryl groups exposed increased 100 per cent when cells were cholesterol enriched compared to cholesterol depleted, the mechanism of cholesterol interaction with this protein remains unclear. The results presented by Nigg and Cherry (1979) suggested the lack of interaction of cholesterol with Band 3, whereas the work of Klappauf and Schubert (1977) suggested this protein interacted strongly with cholesterol. The difference in results may be due to the artificial system used by Klappauf and Schubert as opposed to the natural membrane environment studied by Nigg and Cherry.

A recent study was carried out by Lange et al. (1980) to investigate the effect of cholesterol on the contour and stability of the isolated red cell membrane by cholesterol oxidase digestion. They showed that the cholesterol:phospholipid molar ratio in intact red cell membranes was equal to or just less than 0.8, and that these membranes were

resistant to cholesterol oxidase digestion. Enrichment of the membranes to a molar ratio of 0.9 rendered the entire cholesterol pool available for oxidation. Cholesterol depletion of enriched membranes reversed this susceptibility. These workers suggested that red cell membrane cholesterol is maintained in vivo just below a critical level at which important organizational changes can occur.

Experiments such as those described above have therefore shown that membrane properties can be influenced by the cholesterol content. Studies of a more applied nature have been carried out to investigate the reasons for abnormal membrane architecture occasionally found in the red cells of diseased subjects. Cooper (1969) studied the sera and red cells from three patients with severe liver disease and found abnormal red cells with spiky projections in the blood of these patients. The term "spur cell" has been applied to these bizarre red cells to distinguish them from the morphologically similar but chemically different acanthocytes seen in patients with abetalipoproteinaemia (Ways et al., 1963). Cooper (1972) showed that the unesterified cholesterol content of spur cells averaged 52 per cent higher than in red cells from normal subjects. It was possible that the unusual membrane architecture was due in part to the raised cholesterol content. Cooper et al. (1972) showed that when normal red cells were incubated in spur cell serum, the cholesterol content increased and the cells took on a spur cell like appearance. Conversely, spur cells incubated in normal serum lost cholesterol and took on a normal red cell appearance. In a further investigation, Cooper et al. (1972) analysed the lipoproteins and red cells from patients with alcoholic cirrhosis of the liver. They showed that there was no correlation between serum cholesterol concentration and red cell content of free cholesterol. However, they observed a close relationship between red

cell and serum (or isolated LDL) free cholesterol when considered in relation to the content of phospholipid in each. In view of this close relationship, Cooper et al. (1975) undertook to establish an artificial system that reproduced the cholesterol disproportion in the serum of patients with spur cells in order to study this factor in the absence of other possible influencing factors. These workers prepared cholesterol-phosphatidylcholine dispersions to incubate with red cells in order to alter their cholesterol content *in vitro*. They showed that when normal red cells were incubated with cholesterol-rich dispersions they developed spur cell like morphology concomitant with becoming enriched with cholesterol. The same study showed that spur cells incubated in a cholesterol depleted medium gave rise to morphologically normal red cells which had a normal cholesterol content. Recently Cooper et al. (1978) showed that the membrane fluidity of red cells was decreased as a result of cholesterol enrichment by this *in vitro* technique.

1.4 Platelet Function

Platelets are cytoplasmic fragments of megakaryocytes of the bone marrow (Tavassoli, 1980). They have a longitudinal diameter of about 2 μ and are therefore considerably smaller than red blood cells which have a diameter of approximately 8 μ. Like red cells in humans they are anucleate. Platelet counts in humans normally range from 200-500 x 10^9/litre of blood. Platelets have a half life of about 5 days, are normally disc shaped, and are carried by the blood through the intact blood vessels. When a blood vessel is cut to expose a damaged endothelial lining, the platelets quickly adhere to components which are normally hidden. In this process, platelets undergo a change from their disc shape to produce many long pseudopodia-like extensions from the plasma membrane. The pseudopodia from many platelets interconnect to combine many platelets to form a haemostatic plug at the site of

injury to arrest bleeding. Platelets act in the body as the first line of haemostatic defence. Clotting factors in the plasma also contribute to the formation of the clot to prevent blood loss and to the stabilization of the initial platelet plug. It is believed that several stimuli may activate platelets to aggregate *in vivo*. Collagen revealed by damaged intima may stimulate platelets (Haft, 1979) but it has also been suggested that ADP from damaged red cells may activate platelets in the locality of the wound (Garder et al., 1961). Thrombin is a very potent platelet aggregating agent, and this is an important clotting factor. Thrombin, formed from prothrombin (which circulates in the blood) by the action of prothrombin activator, induces the proteolysis and polymerization of fibrinogen to fibrin threads. Fibrin plays an important role in the stabilization of a newly formed clot (see for example Jackson and Nemerson, 1980).

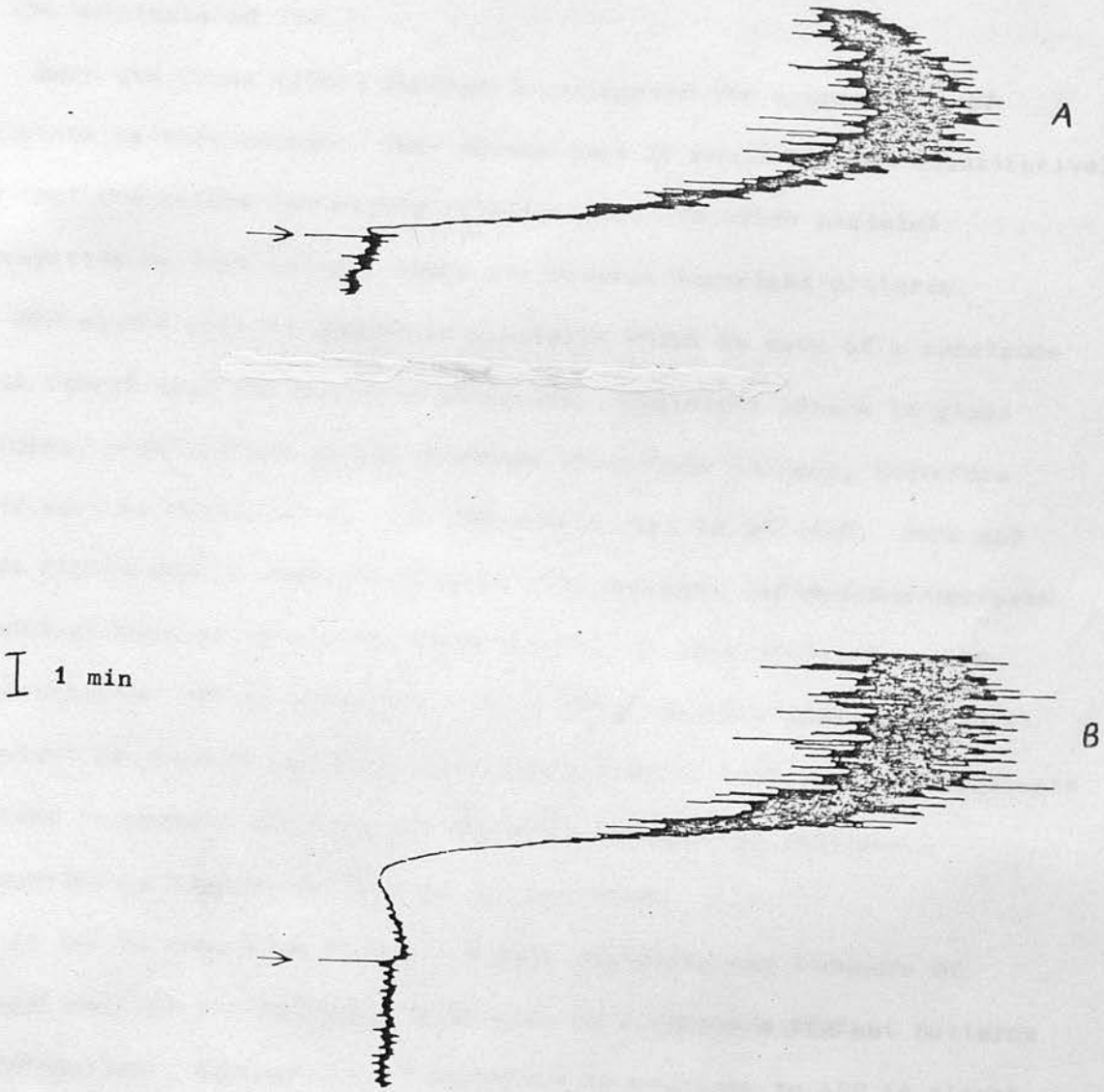
The process of platelet aggregation has been extensively studied *in vitro*. Researchers have become interested in this phenomenon because platelet aggregation leads to the formation of thrombi. Thrombi consist largely of platelets, and platelets are therefore implicated in the development of thrombosis and atherosclerosis. The method commonly used to study platelet aggregation *in vitro* is the turbidimetric assay. This was developed by Born (1962) and he used this method to study the aggregation and disaggregation of platelets when stimulated with ADP. The optical density of platelet rich plasma (PRP) was recorded at 600 nm when stimulated with ADP. Born showed that the optical density of a sample of PRP decreased rapidly after addition of ADP. Figure 1.3 shows a typical aggregation trace of rat PRP recorded by this method. With a sufficiently low concentration of ADP, the optical density can be observed to increase again, the whole process taking 2 to 3 minutes.

Figure 1.3 Aggregation of rat platelets

A Aggregation induced by ADP (0.12 µM)

B Aggregation induced by collagen (2 µg/ml)

Figure 1.3. Aggregation of rat platelets



The decrease in optical density occurs as a result of the formation of platelet aggregates such that the number of individual platelets in suspension decreases. The increase in optical density concomitant with disaggregation is a result of dispersion of aggregates such that the number of individual platelets in suspension increases. Disaggregation may be complete so that the optical density returns to the same level as the unstimulated PRP.

Born and Cross (1963) further investigated the aggregation of platelets by this method. They showed that it could be used quantitatively and that the method was highly reproducible. To study platelet aggregation by this method, there are several important criteria. The PRP sample must be placed in a cuvette which is made of a substance which itself does not activate platelets. Platelets adhere to glass surfaces, possibly due to the presence of surface changes, therefore glass must be siliconised. The PRP sample must be stirred. Born and Cross (1963) showed that stirring at 1000 rpm gave the maximum decrease in optical density to a given dose of ADP. In this study, Born and Cross obtained PRP by centrifugation of blood samples taken into anti-coagulant to prevent clotting. Concentrations of suitable anticoagulants are used to prevent clotting but which do not inhibit platelet aggregation as studied by this <u>in vitro</u> method.

It can be seen from Figure 1.3 that the different inducers of aggregation, ADP and collagen, give rise to slightly different patterns of aggregation. Aggregation of platelets in response to ADP is almost instantaneous, whereas aggregation in response to collagen occurs after a time lag of 1 to 2 minutes. Aggregation to both agents is preceeded by an apparent increase in optical density. This is more obvious with the collagen induced aggregation. This is a transient swelling of platelets from the normal disc shape. The small oscillations of the

PRP sample observed prior to stimulation is caused by oscillation of the disc shaped platelets being stirred in the light path. This oscillation is reduced because the platelets swell and become spherical. Following the swelling phase a rapid decrease in optical density occurs which is due to the formation of pseudopedia and the aggregation of platelets as mentioned above. Platelets contain various components which are secreted when activated by a suitable stimulus. In particular, platelets contain dense granules and α-granules. The sequence of events occurring after stimulation is described as the basic platelet reaction (Holmsen et al., 1977). This sequence is shape change, aggregation, dense granule secretion and α-granule secretion. The stage at which this sequence terminates depends on the strength of the stimulus.

In human platelets, aggregation in vitro occurs in two waves. Primary, or first wave aggregation is reversible. With a strong stimulus disaggregation does not occur, and second wave irreversible aggregation proceeds, with the secretion of granules. Rat platelets do not have a biphasic aggregation pattern. They undergo a single wave of aggregation which is reversible with a low dose of inducer, and irreversible with a high dose. Most aggregating agents can induce dense granule secretion, but only strong agents induce secretion of α-granules. Collagen is believed to be a strong aggregating agent, whereas ADP is a weak agent and does not induce α-granule secretion (Holmsen et al., 1977), but it can induce the second wave of aggregation in human platelets. In human platelets, dense granules contain ADP and serotonin which may contribute to inducing aggregation of neighbouring platelets. α-granules contain fibrinogen and various acid hydrolases. Fibrinogen from platelets may be important in collagen induced aggregation (Solum and Stormorken, 1965). In view of the formation of pseudopodia by aggregating platelets, the possibility of the involvement of a contractile system was investigated

(for example Luscher and Bettex-Galland, 1972). The system is believed to be similar to that of muscle cells, except that there appear to be two pools of contractile proteins in platelets, one in the membrane and the other in the cytoplasm.

Platelets are stimulated to aggregate by a great variety of agents. These may be subdivided into three classes (Luscher and Massini, 1975), namely proteolytic enzymes, "large molecules" or complexes of molecules, and low molecular weight substances. Thrombin is a proteolytic enzyme which is a strong platelet stimulator. Collagen has been regarded as an example of a "large molecule". It is necessary that the polymerised form of collagen is present to stimulate platelets. The carbohydrate moiety of the collagen molecule is not considered important in platelet activation (Muggli and Baumgartner, 1973). However the ordered, fibrillar, quaternary structure of collagen is required for the stimulation of platelets to aggregate (Gordon, 1979). Low molecular weight molecules include adrenaline, 5-hydroxytryptamine and ADP. These substances are released by activated platelets. Indeed, collagen induced stimulation of rabbit platelets was reported to act by causing the release of ADP from the platelets themselves (Hovig, 1963). It has been shown by Haslam (1964) that ADP was of importance in the aggregation of platelets by utilizing pyruvate kinase and phosphoenol pyruvate to remove low concentrations of ADP. Haslam showed that for thrombin, trypsin and ADP induced aggregation, the aggregation decreased with increasing concentrations of pyruvate kinase and phosphoenolpyruvate. This suggested that ADP played a very significant role in platelet aggregation.

It was suggested by Luscher and Massini (1975) that there may be a common pathway of membrane reactivity leading to platelet activation, and for many of the events of the basic platelet reaction, the availability of calcium ions within the cell is essential. It has been well

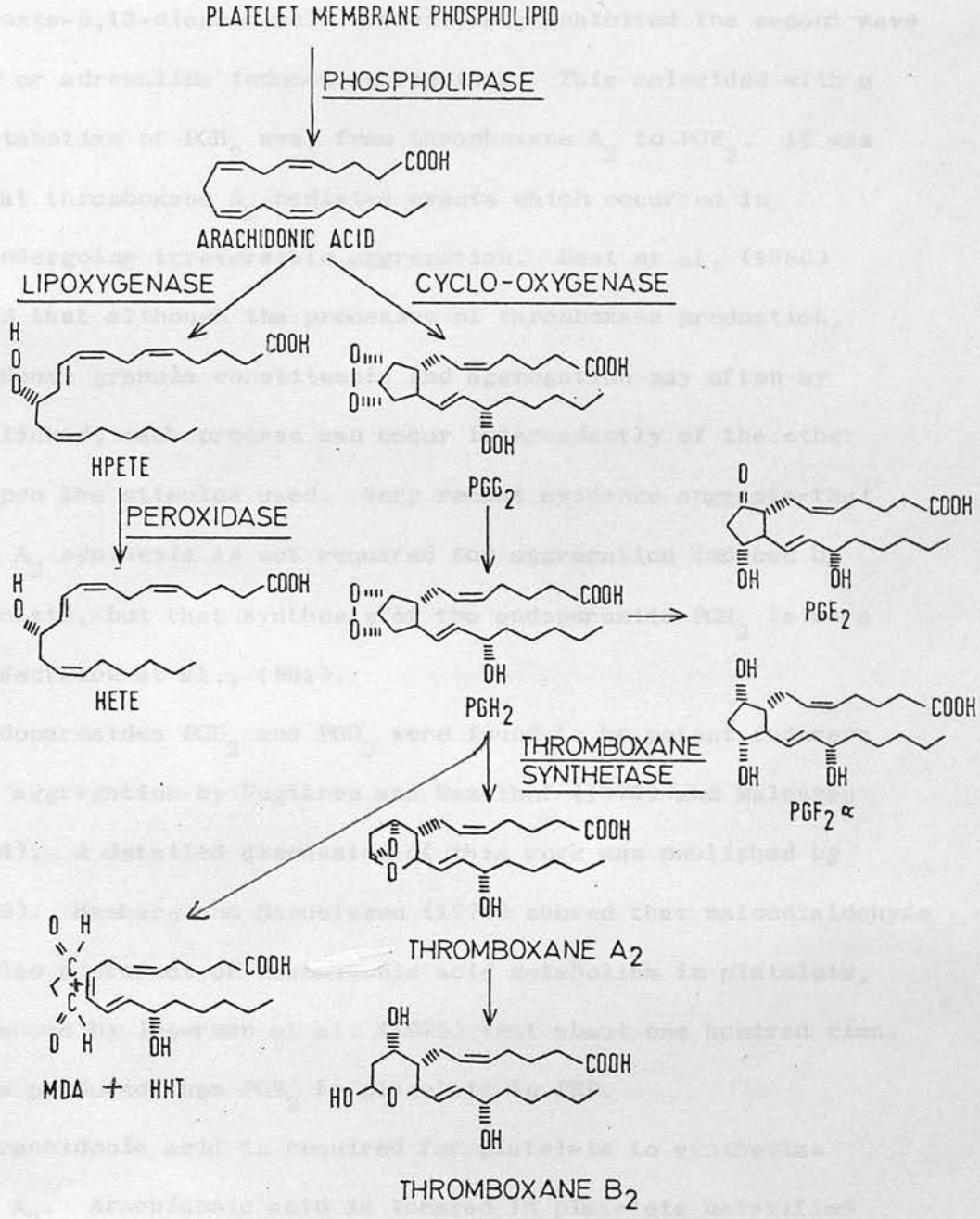
established that an increase in the platelet cAMP level will prevent the onset of platelet activation. It is possible that cAMP is an important secondary messenger which maintains platelet integrity. It has been suggested that cAMP plays this role by preventing the mobilization of calcium from, or by inducing the sequestration of calcium by internal organelles (Gerrard et al., 1978). Haslam et al. (1978) suggested that cAMP has a "unidirectional" control of platelet function.

Much research has been carried out to investigate the factors involved in the irreversible aggregation of platelets. There is much evidence that ADP is necessary for this process to occur, but the synthesis of various prostaglandins has also been shown to take place concomitantly with irreversible aggregation. Thrombin induced aggregation has been shown to occur without prostaglandin synthesis however (Smith et al., 1975), but Marcus (1978) reviewed much work which demonstrated that products of arachidonic acid metabolism are important mediators of platelet function.

1.5 Arachidonic Acid Metabolism in Platelets

The metabolic pathway of arachidonic acid in platelets is summarized in Figure 1.4. It has been shown that human platelets synthesize prostaglandins E_2 and F_2 during aggregation induced by various agents (Smith and Willis, 1970 and Smith et al., 1973). Hamberg et al. (1975) identified a new biologically active compound which was synthesized by platelets from prostaglandin endoperoxides which had in turn been synthesized from arachidonic acid. This compound was called thromboxane A_2 and was formed by platelets which were incubated with arachidonic acid to induce irreversible aggregation and serotonin release. This work complemented previous work by Hamberg et al. (1974) who showed that human platelets produced equimolar amounts of HETE, HHT and thromboxane B_2 (see Figure 1.4). The lipoxygenase enzyme, which

Figure 1.4. Metabolism of arachidonic acid in platelets



synthesises HETE from arachidonic acid, was identified by Nugteren (1975). Approximately 100x more of these compounds were synthesized than of either PGF_2 or PGE_2. Gorman et al. (1977a) showed that platelets incubated with a specific thromboxane A_2 synthetase inhibitor (9,11-azoprosta-5,13-dienoic acid) selectively inhibited the second wave of both ADP or adrenaline induced aggregation. This coincided with a shift of metabolism of PGH_2 away from thromboxane A_2 to PGE_2. It was possible that thromboxane A_2 mediated events which occurred in platelets undergoing irreversible aggregation. Best et al. (1980) demonstrated that although the processes of thromboxane production, release of dense granule constituents and aggregation may often by intimately linked, each process can occur independently of the other depending upon the stimulus used. Very recent evidence suggests that thromboxane A_2 synthesis is not required for aggregation induced by various agonists, but that synthesis of the endoperoxide PGH_2 is more important (Westwick et al., 1981).

The endoperoxides PGH_2 and PGG_2 were found to be potent inducers of platelet aggregation by Nugteren and Hazelhof (1973) and Malmsten et al. (1974). A detailed discussion of this work was published by Marcus (1978). Hamberg and Samuelsson (1974) showed that malondialdehyde (MDA) was also a product of arachidonic acid metabolism in platelets, and it was shown by Ingerman et al. (1975) that about one hundred times more MDA was produced than PGE_2 by platelets in PRP.

Free arachidonic acid is required for platelets to synthesize thromboxane A_2. Arachidonic acid is located in platelets esterified to the sn-2 hydroxyl group of the glycerol moiety of platelet membrane phospholipids. No free arachidonic acid is available in normal platelets (Marcus et al., 1969). There are believed to be two important enzymes responsible for the release of arachidonic acid

from platelet phospholipids (Billah et al., 1980). Firstly a phospholipase A_2 exists in platelets to hydrolyse the sn-2 ester bond to provide free arachidonic acid and the corresponding lysophospholipid. Secondly, a phospholipase C enzyme exists which is specific for phosphatidylinositol, removing the polar head group to form the diacylglycerol (Bell et al., 1979). This diacylglycerol is then either phosphorylated to phosphatidic acid by a diacylglycerol kinase (Billah et al., 1979) or hydrolysed by diacylglycerol lipase (Bell et al., 1979) to provide free arachidonic acid. Arachidonic acid is made available by either pathway for oxygenation by a cyclooxygenase to ultimately form thromboxane, or by a lipoxygenase to HETE (Nugteren, 1975) (Figure 1.4).

A mode of action for the proaggregating effect of thromboxane A_2 has been proposed. It is believed to act as a calcium ionophore, directly transporting calcium from the platelet dense tubular system to the cytoplasm to activate the contractile proteins (Gerrard et al., 1976). Calcium is critical for the activation of phospholipase A_2, and Gerrard et al. (1978) suggested that calcium may play an important role in thromboxane A_2 synthesis. In their model, Gerrard et al. suggested that the enzymes involved in arachidonic acid metabolism are located in close proximity to one another in the dense tubular system. The role of cAMP was considered to be one of promoting Ca^{2+} uptake into the dense tubular system away from these enzymes thereby inhibiting platelet activation and prostaglandin synthesis (Gerrard et al., 1977).

While much research was being carried out to investigate arachidonic acid metabolism by blood platelets, Moncada et al. (1976) discovered a product of arachidonic acid metabolism in vascular tissue. This was initially named PGX, and was observed to induce vasodilatation of vascular tissue and to inhibit ADP induced aggregation of human platelets

(Gryglewski et al., 1976). Structure determination and total organic synthesis of PGX was achieved by Johnson et al. (1976) and these workers named the compound prostacyclin, PGI_2. At about the same time Needleman et al. (1977) observed that arachidonic acid induced a relaxation response when added to bovine or human coronary artery strips. This group were unknowingly observing the effects of PGI_2. PGI_2 was observed to inhibit platelet aggregation and to stimulate platelet adenylate cyclase to synthesize cAMP(Gorman et al., 1977b; Tateson et al., 1977).

Since the discoveries of thromboxane A_2 and PGI_2, it has been postulated that an intricate regulation of platelet aggregation exists (Gorman, 1979). A "reciprocal regulation" of cAMP levels was suggested between the endoperoxide-thromboxane A_2 system presumed to inhibit adenylate cyclase and the PGI_2 mediated stimulation of adenylate cyclase. The actions of thromboxane A_2 and PGI_2 are not restricted to platelets, but affect the cardiovascular system also. PGI_2 inhibits platelet aggregation, relaxes smooth muscle of the coronary arteries, and reduces blood pressure. Thromboxane A_2 opposes all the effects of PGI_2, inducing platelet aggregation, constricting arteries and thereby raising blood pressure (Bailey, 1979). Both these compounds are very labile, and in view of the antithrombotic action of PGI_2, researchers have been attempting to synthesize a stable analogue which would have clinical use. It has been shown that platelets from particular patients synthesized significantly more prostaglandin endoperoxides than platelets from normal subjects. The groups of patients studied were subjects with post-operative deep vein thrombosis and subjects with arterial thrombosis (Lagarde and Dechavanne, 1977). Since platelets have long been implicated in the pathogenesis of atherosclerosis and thrombosis (Constantinides, 1976), researchers have looked for therapeutic agents to inhibit platelet activity in some way (Weiss, 1976).

To assay platelet thromboxane A_2 production in order to assess platelet function, two common methods have frequently been used. Thromboxane A_2 is very unstable, and rapidly transforms to the more stable metabolite thromboxane B_2 which is quantitated by radioimmunoassay. Platelets produce equimolar amounts of thromboxane B_2 HHT and MDA, and some researchers have assayed MDA production as an indicator of thromboxane A_2 synthesis. The method was originally described by Placer et al. (1966) and was adapted for platelet MDA assays by Stuart et al. (1975). The method involves the detection of the chromagen produced by the complex formed between MDA and thiobarbituric acid by light absorbance at a wavelength of 532 nm. A fluorometric assay of this complex has more recently been developed for the detection of smaller quantities of MDA (McMillan et al., 1977).

MDA has been shown to be produced by platelets during the second phase of aggregation by human platelets (Macfarlane et al., 1977). Thromboxane synthetase, the enzyme responsible for conversion of PGH_2 to thromboxane A_2 has been shown to catalyze the formation of MDA (McMillan et al., 1978). Recently these methods have been used to evaluate platelet thromboxane A_2 production in platelets from diseased subjects.

1.6 Hypercholesterolaemia

As mentioned previously, Cooper et al. (1972) showed that the cholesterol to phospholipid molar ratio of the red blood cell membrane was apparently influenced by the cholesterol:phospholipid ratio of the plasma constituents and subsequently showed that the cholesterol: phospholipid molar ratio of LDL correlated well with the ratio found in red cell membranes(Cooper, 1977). Carvalho et al. (1974a) examined platelets from patients who were known to be susceptible to atherosclerosis, having been diagnosed with type lla hyperbetalipoproteinaemia

(type lla). The common features in this disease are a high plasma cholesterol level (often over 400 mg per 100 ml blood), and a high plasma LDL level. Hypercholesterolaemia has frequently been suggested as a factor involved in the pathogenesis of atherosclerosis and thrombosis (see for example Marx, 1976). In the disease cholesterol carried by LDL cannot enter extrahepatic cells and thereby inhibit the function of HMGCoA reductase (Brown and Goldstein, 1976, 1980). Because of the lack of inhibition, this enzyme is very actively synthesizing cholesterol which enters the blood. Type lla hyperbeta-lipoproteinaemia is a genetic disorder displaying the features of autosomal dominance and sufferers are prone to early age severe atherosclerosis (Lees et al., 1973).

Carvalho et al. (1974a) observed that platelets from type lla subjects were hyper-sensitive to the aggregating agents ADP, adrenaline and collagen. These platelets aggregated with significantly lower concentrations of these agents compared to platelets obtained from normal subjects. Platelets from type lla subjects have therefore been investigated by several research groups. Bizois et al. (1977) showed that platelets from type lla subjects produced significantly more thromboxane B_2 from radiolabelled arachidonic acid compared to normal platelets. Tremoli et al. (1979a,b) showed that platelets from type lla subjects showed enhanced MDA production and enhanced thromboxane B_2 production induced by arachidonic acid compared to platelets from normal subjects. This is an interesting observation because it was hypothesized by Fogelman et al. (1980) that LDL may be modified *in vivo* by MDA released from blood platelets or produced by lipid peroxidation at the site of arterial injury. These workers showed that this modified LDL was taken up by human monocyte-macrophages more readily than native LDL, resulting in enhanced cholesteryl ester accumulation in these cells.

Bennett et al. (1974) reported that platelets from type lla subjects had a raised cholesterol to phospholipid ratio. This was confirmed by Shattil et al. (1977) and these platelets were demonstrated to be hypersensitive to aggregating agents. The ratio was 7 per cent higher than in platelets from normal subjects. It was shown by Colman et al. (1976) that halofenate treatment for type lla subjects could reduce the enhanced sensitivity of platelets to aggregating agents. This was found to be a more effective therapy than administration of clofibrate to type lla subjects. Clofibrate did however reverse the hypersensitivity of platelets from type lla subjects (Carvalho et al. (1974b)) but to a lesser extent.

Shattil et al. (1975) used the in vitro method to alter platelet cholesterol content which Cooper et al. (1975) used for alteration of the cholesterol content of red blood cells. Liposome suspensions made cholesterol poor, normal or rich were incubated with platelets to deplete, maintain or enrich them with cholesterol. Enrichment of platelets with cholesterol enhanced their sensitivity to ADP and adrenaline induced aggregation, whereas cholesterol depletion had the reverse effect. This study by Shattil et al. (1975) discounted the possibility of any plasma factor mediating the platelet hypersensitivity because the platelets were isolated from healthy donors. The results showed that a high serum cholesterol level may induce hypersensitivity by enriching platelets with cholesterol. In another study, it was shown that the cholesterol content affected the microviscosity of the platelet membrane (Shattil et al., 1976). Using diphenyl hexatriene it was shown that an increased cholesterol content increased the rigidity of the platelet membrane. A decreased cholesterol content caused the membrane to become more fluid.

An explanation for the enhanced sensitivity of platelets with raised cholesterol to phospholipid molar ratios remained obscure. Sinha et al. (1976) reported that altered platelet cholesterol content by this in vitro method caused changes in cAMP metabolism. They showed that basal adenylate cyclase activity was enhanced in cholesterol enriched platelets. Stimulation of adenylate cyclase activity by PGE, and NaF was lost in cholesterol enriched platelets compared to control platelets. It was suggested that cholesterol enrichment induced a loss of response due to a physical effect of cholesterol on platelet membrane phospholipids and hence on adenyl cyclase. In a subsequent study by Insel et al. (1978) it was shown that alteration of platelet cholesterol content did not result in changes in the level of adrenaline bound to its receptor, and that changes in adenylate cyclase activity seen previously by Sinha et al. (1976) did not occur.

More recently it was shown by Stuart et al. (1980a) that human platelets with altered cholesterol content demonstrated correspondingly altered thromboxane B_2 production. Platelets enriched with cholesterol by the method of Shattil et al. (1975) were shown by Stuart et al. to synthesize more thromboxane B_2 when stimulated with thrombin compared to cholesterol depleted platelets. A recent study by Worner and Patscheke (1980) demonstrated that platelets enriched with cholesterol in the same way showed enhanced arachidonic acid metabolism. It has therefore become clear that cholesterol in the platelet membrane may influence the activities of systems which catalyze the release and subsequent metabolism of arachidonic acid. Release of arachidonic acid from platelet membrane phospholipids, as described above, could be a very important rate limiting step in the provision of substrate for thromboxane A_2 synthesis. This initial step, probably regulated by phospholipase A_2 and phospholipase C (Billah et al., 1980) has not been investigated satisfactorily in cholesterol-enriched platelets.

Much research has been carried out on atherosclerosis induced in animals. In these animal model systems platelet function has been investigated in order to attempt to explain the pathogenesis of the disease in humans. This is discussed more fully in Chapter 6.

The hypersensitivity of platelets induced by cholesterol enrichment required further research to evaluate what other platelet functions were affected. The purpose of the present project was to continue research in this area.

1.7 Experimental Approach of Project Described in this Thesis

Cholesterol is an important component of the platelet membrane. The membrane performs a highly specialized function which allows the aggregation of platelets in the process of haemostasis. In the present studies the role of cholesterol in platelet membranes has been examined. In order to carry out these investigations, it was necessary to develop methods to alter the membrane cholesterol level. There were two possible approaches:

1. To incubate platelets in a medium which would selectively alter the cholesterol content of rat and human platelets *in vitro*. A suitable method was considered to be that developed by Shattil et al. (1975) as mentioned above. This method had the advantage of altering cholesterol content only, without affecting other factors.

2. To attempt to increase the platelet cholesterol content *in vivo* by feeding a group of rabbits a diet rich in cholesterol. This would be expected to raise the plasma cholesterol level thus presenting the circulating platelets with a cholesterol enriched medium. A group of lop-eared rabbits were used in these experiments (see Chapter 6).

Assessment of platelet function was carried out with particular reference to membrane associated events. Aggregation was studied using an aggregometer by the method of Born and Cross (1963). The effect of

alteration of cholesterol content on phospholipase A_2 activity in platelets has not previously been investigated satisfactorily. This enzyme is, at least in part, responsible for the release of arachidonic acid, and it was particularly pertinent to investigate the effect of cholesterol on the activity of this membrane bound protein. A phospholipase A_2 assay was developed in order to determine whether platelet hypersensitivity to aggregating agents is mediated by alteration of the activity of this enzyme.

To investigate further the requirement of a sterol of specific molecular dimensions for membrane integrity, cholesterol analogues of shorter length side chains were incorporated into platelet membranes in vitro. The platelet membrane architecture rapidly changes on stimulation. Cholesterol is suspected to fit precisely into membranes, and it was considered that a membrane performing such a specialized function may depend upon components which contribute to the maintenance of its integrity. This study was intended to investigate this possibility and was a continuation of the work of Craig (1978) who investigated the effect of cholesterol analogues in artificial membranes, and cholesterol metabolising systems.

Chapter 2

Materials and Methods

2.1 Animals and Diets

Male and female rats of the Wistar strain, weighing approximately 150 g, were obtained from the Bush Small Animal Breeding Centre of Edinburgh University. They were fed a normal stock diet and water ad libitum. Experiments involving the use of rabbits were carried out at ICI Pharmaceuticals Division, Alderley Park, Cheshire. White male Lop-earned rabbits (3 kg) were fed a stock diet or the same diet supplemented with cholesterol (0.5 per cent w/w) to induce hypercholesterolaemia.

The normal stock diet fed to rats consisted of 70 per cent wholemeal flour, 25 per cent skimmed milk powder and 5 per cent dried yeast. The stock diet fed to rabbits consisted of Labsure animal diet CRB pellets which was supplied by Christopher Hill Group Ltd., Poole, England. A complete breakdown of constituents of this diet is provided in Table 2.1.

Human subjects

Human subjects providing blood samples for experimental purposes were requested not to take any medications for at least ten days prior to donation.

2.2 Blood Sampling

1. Rats: Rats were anaesthetized with ether, the thorax was cut open from the ventral side. Fatty tissue around the heart was removed, and blood was removed from the right ventricle using a syringe. A 20 ml syringe was used with a 21 g needle which had been rinsed with anticoagulant. The anticoagulant used was trisodium citrate (3.8 per cent w/v) to a final volume ratio of 1:9 volumes of blood. Approximately 10 ml of blood was obtained from a rat. The blood sample was put in a 10 ml plastic screw top blood sample tube and inverted gently twice.

Table 2.1. Labsure animal diet CRB pellets, supplied by Christopher Hill Group Ltd., Poole, Dorset, England

1. Analysis	Per cent by weight	Raw materials
Crude oil	1.92	Wheat
Crude protein	16.1	Maize
Crude fibre	14.0	Oat Feed
Calcium	1.1	Soya
Phosphorous	0.7	Fish meal
Available Phosphate	0.52	
NaCl	0.75	
Metabolisable Energy	1900 kcal/kg	

2. Trace elements	Parts per million
Mn	125
Cu	7
Co	0.4
Fe	30
I	1.3
Mg	102

3. Vitamins Added	
A	8000 i.u.
D_3	1000 i.u.
B_2	8 mg
Nicotinic acid	50 mg
Pantothenic acid	12 mg
B_{12}	12 µg
E	100 i.u.
K	10 mg
Folic acid	10 mg
Choline	200 mg
B_1	4 mg
B_6	6 mg

2. Rabbits: Blood samples were taken from rabbits by Mrs. S. Jones at ICI Pharmaceuticals Division. Rabbits were restrained, and a 19 G needle attached to a length of plastic tubing was inserted into the ear artery. Blood was allowed to flow freely into a 50 ml plastic centrifuge tube containing 3.8 per cent (w/v) trisodium citrate to a volume ratio of anticoagulant of 1:9 volumes of blood.

3. Human subjects: Units of blood were obtained from the Edinburgh Blood Transfusion Service. Blood was collected into citrate-phosphate-dextrose anticoagulant under standard BTS conditions. Blood samples collected from subjects by Dr. M. Stone at the Leigh Health Centre were taken into 3.8 per cent (w/v) trisodium citrate (1:9 volumes blood). The blood was collected from the antecubital vein.

2.3 Platelet Preparation

Blood samples were centrifuged at 100 g for one or two periods of 10 min in an MSE bench centrifuge to sediment the red blood cells. The top layer of PRP was collected. PRP from rats was pooled prior to experimentation. In experiments using human blood, PRP from different subjects was never mixed.

2.4 Platelet Counting

Platelet counts were carried out on PRP samples, samples of PRP-liposome mixtures or resuspended platelet samples. In Edinburgh, platelet samples were kindly counted by the Blood Coagulation Unit, Royal Infirmary of Edinburgh using a thrombocytometer (Coulter Electronics). At ICI Pharmaceuticals Division, platelet samples were counted using a Coulter Counter type B model (Coulter Electronics) fitted with a 50 μ orifice (Sipe and Cronkite, 1962). 6.6 μl of the platelet sample was diluted in 17 ml isoton (Coulter Electronics) for the purpose of counting. Three determinations were made for each sample, and the mean was calculated.

2.5 Preparation of Egg Yolk Lecithin

Phosphatidylcholine was prepared from egg yolks by the method of Pangborn (1951), which involved an initial acetone extraction to remove all the neutral lipids leaving a cake of protein and phospholipids. A 95 per cent ethanol extraction of the cake separated the protein from phospholipids, which were subdivided by a combination of solvent extraction methods and the formation of cadmium adducts of the phosphatides. Sphingomyelin was retained with the phosphatidylcholine during this preparation but was eventually removed by precipitation from a cold ether solution of PC. A sample was subjected to thin layer chromatography developed in chloroform:methanol:water (65:35:5 v/v). Staining in iodine vapour gave a single spot which was identical to a similarly treated sample of authentic PC thereby confirming the absence of other phospholipids.

2.6 Liposome Preparation

Liposome suspensions were prepared by a method similar to that described by Cooper et al. (1975), and by Shattil et al. (1975). L-α dipalmitoyl phosphatidylcholine (DPPC) 40 mg and unesterified cholesterol or cholesterol analogues in amounts up to 50 mg were mixed and dissolved in chloroform (3 ml) in a round bottom flask. The organic solvent was removed by warming the flask while turning it on a rotary evaporator. The residue remained as a thin lipid film coated on the bottom of the flask. All the remaining organic solvent was removed by placing the flask in a vacuum dessicator for 30 min.

10 ml of modified Tyrode's buffer was added to the lipid film. This buffer contained 0.137M NaCl, 2.7 mM KCl, 1.19 mM $NaHCO_3$, 0.42 mM $NaH_2PO_4.2H_2O$ and HCl to adjust the pH to 7.4. The flask containing the lipid film was held under a stream of hot water, and the Tyrode's buffer, preheated to 50^{o}C was then added. The flask was vigorously shaken under the stream of hot water. After 3 min, all the lipid was removed from the surface of the glass, and a cloudy suspension of lipid was formed. The mixture was sealed under nitrogen and sonicated

by suspending the flask in a sonicating water bath (Dawe Instruments Ltd.) of constant power output at 55°C for 3-4 hours.

At the end of this sonication period the liposome preparations were transferred to plastic centrifuge tubes (50 ml) and centrifuged for 30 min at 22,000 g in a Beckman J2-21 centrifuge at 20°C to remove undispersed lipid which formed a pellet. The supernatant was decanted and centrifuged again under the same conditions to ensure that all the undispersed lipid had been removed. The liposome suspensions were transferred to 10 ml screw top sample tubes. Glucose and albumin were added to final concentrations of glucose (5 mM) and albumin (3.5 mg/ml). Liposome suspensions were optically clear, and were used within 15 hours of preparation.

The following liposome suspensions were prepared for use in experiments described here. In all cases, 40 mg of DPPC was used.

1. Cholesterol-poor liposomes were prepared with no cholesterol added. This medium was used to deplete blood platelets of cholesterol.

2. Cholesterol-normal liposomes were prepared with 12.5 mg of cholesterol. This medium was used to maintain a normal level of cholesterol in platelets.

3. Cholesterol-rich liposomes were prepared with 50 mg of cholesterol. This medium was used to enrich platelets with cholesterol.

Liposomes were also prepared containing cholesterol analogues. Suspensions of these liposomes were used to deliver the analogues to the platelets.

Δ^5 pregnene-3β-ol (C_{21})-rich liposomes were prepared using 50 mg of this sterol with the DPPC. Δ^5 cholen-3β-ol (C_{24})-rich liposomes were prepared using 40 mg of this sterol. Also 40 mg of 27 nor Δ^5 cholestene-3β-ol (C_{26}) was used with DPPC to make C_{26}-analogue rich liposomes.

2.7 PRP-liposome Incubation Procedure

PRP was mixed with an equal volume of liposome suspension (normally 5 ml + 5 ml) in a 10 ml screw top sample tube. Samples were incubated in a water bath at 37°C generally for a period of 3 hours or longer where stated. Samples were removed at half hour intervals and gently inverted to ensure mixing of platelets and liposomes. This method is essentially the same as was used by Shattil et al. (1975).

2.8 Analysis of Platelet Protein and Lipids

Platelets were separated from plasma constituents and liposomes by centrifuging 2-4 ml of the sample of PRP at 4800 g for 15 min in a Beckman J2-21 centrifuge. The supernatant was discarded, and the platelets were resuspended in 4 ml of platelet washing buffer containing NaCl 0.15M, Tris 10 mM, EDTA 0.1 mM pH 7.4. This was repeated twice more. Platelet samples were finally resuspended in 2 ml of the same buffer. 50 µl samples were taken for triplicate protein estimation by the method of Lowry (1951). Lipids were extracted from the remaining sample by the method of Bligh and Dyer (1959). The dried lipid extract was dissolved in chloroform, and aliquots were analysed for phospholipid by the method of McClare (1971) and cholesterol was analysed using cholesterol oxidase from Nocardia species (see below). When samples contained a mixture of cholesterol and cholesterol-analogue, sterol composition was determined by gas liquid chromatography with a Pye 104 model using the same conditions as described by Craig (1978) (see below).

1. Phospholipid assay by the method of McClare (1971)

In this method, triphenyl phosphine was used to make up standards of known phosphate content. A colorimetric reagent was freshly prepared containing 8 per cent perchloric acid, 1 per cent ammonium molybdate and 0.2 per cent ascorbic acid. The samples of unknown phospholipid content

were blown down to dryness, and all traces of organic solvent were removed by placing the samples in a vacuum dessicator for 30 min. The extracts were then digested for 1 hour by boiling with 0.5 ml of perchloric acid at approximately 200°C. When the samples had cooled, 9.5 ml of the colorimetric reagent was added to each digest tube which was then covered, inverted twice and incubated for 40 min in a waterbath at 50°C. The phosphate content was measured by absorbance against a reagent blank at 825 nm and converted to µg phospholipid by multiplication by 25. This method detected quantities of phosphorus in the range 1-7 µg.

2. Cholesterol assay using cholesterol oxidase

Cholesterol oxidase is an oxidoreductase which reacts specifically with Δ^4-or Δ^5-3β-hydroxycholestanes. Cholesterol is metabolised to Δ^4-cholestenone by this enzyme with the release of hydrogen peroxide as a by-product. This is measured by monitoring at 500 nm the pink colour produced by a hydrogen peroxide, phenol, 4-aminophenazone, peroxidase complex (Trinder, 1969). The presence of high levels of peroxidase activity and sodium azide ensure that catalase does not interfere with colour production.

Samples of lipid extracts to be analysed for cholesterol were dissolved in 100 µl of isopropanol. To these, 2.5 ml of a solution was added prepared by mixing equal volumes of 0.4M Tris HCl buffer (pH, 7.0) containing phenol (0.144 per cent w/v) and Triton X-100 0.5 per cent, and 0.05M tris HCl buffer (pH, 7.0) containing 4-aminophenazone (0.022 per cent, w/v). Cholesterol oxidase was added to each sample (0.025 E.U.) and the reaction proceeded for 15 min at 37°C in a shaking waterbath. The solutions were read against a reagent blank, correcting for turbidity, and the absolute amounts of cholesterol present were determined by comparison with standards. This assay detected quantities of cholesterol in the range 10-100 µg.

3. Cholesterol and cholesterol-analogue assay by gas liquid chromatography

After removal of a sample for phospholipid analysis, the lipid extracts dissolved in chloroform were passed through a silica gel (kieselgel H60) column to remove the remaining phospholipids. The sterol extracts were collected in the eluate, and the volume of chloroform used to ensure the highest recovery was at least two bed volumes. The resulting sterol extracts were then blown to dryness under nitrogen, and finally resuspended in redistilled acetone. Small aliquots were taken for estimation of recovery. Pregnenolone acetate was added to each sample as an internal standard. Samples were then applied to a column of 100-120 mesh gas chrom Q on which was adsorbed 1 per cent SE30. With the column oven at 235°C, the injection heater at 300°C and a nitrogen flow rate of 30 ml min^{-1} the amounts of the sterols present in the sample were determined. This quantitation was achieved by comparing the peak height ratios for the unknown cholesterol and known pregnenolone acetate with standard mixtures of the two components. Table 2.2 shows the time interval after injection of the sample on the column that each sterol was eluted and detected.

Recovery of lipids extracted and processed as described above, was determined by adding a small quantity of [4-^{14}C] cholesterol (50 mCi/mmol) to the platelets prior to extraction with organic solvents. A small aliquot of the final solution of the lipid extract was counted in a vial containing 5 ml of scintillant (20 g PPO, 150 mg POPOP, in 5 litres toluene) in a Packard Tri-carb scintillation counter.

Table 2.2. Gas liquid chromatographic analysis of sterols

Sterol	Elution Time (mins)
C_{21}-analogue	4
C_{24}-analogue	10
Pregnenolone acetate	11
C_{26}-analogue	19
Cholesterol	22

Settings of Pye 104 Gas Liquid Chromatograph.

The elution times show the period after applying a sample to the column.

Injection heater = $300^{o}C$

Oven = $235^{o}C$

N_2 flow rate = 30 ml min^{-1}

Air flow rate = 30 ml min^{-1}

2.9 Platelet Aggregation

Platelet aggregation was studied by the method of Born and Cross (1963). An aggregometer (Payton Associates Ltd., Hamilton, Canada) was used to study the optical density changes of PRP samples, PRP-liposome samples, and resuspended platelet samples. Samples were tested for aggregation with ADP or collagen. Optical density changes were recorded on a Servoscribe chart recorder (Smiths Industries Ltd., U.K.).

Corresponding platelet poor plasma (PPP) samples were prepared for each platelet sample to be tested for aggregation by centrifuging 1 ml of the PRP sample at 1000 g for 15 min in a MSE bench centrifuge. The supernatant PPP was pipetted into a siliconised cuvette containing a siliconised stirrer. For aggregation testing of PRP samples, 0.25 ml samples were added to siliconised cuvettes each containing a siliconised stirrer.

Aggregation was tested and assessed by a modification of a method described by Dreyfus and Zahavi (1973). Using the aggregometer sensitivity controls, the deflection of the chart recorder pen was adjusted so that 1) the deflection when a sample of PRP was placed in the light beam and stirred at 1000 rpm was 0 per cent and 2) the deflection when a sample of PPP was placed in the light beam was 100 per cent. The distance of pen travel between PRP and PPP was adjusted so as to be the same for all samples tested. The percentage maximum extent of aggregation was calculated thus:

$$\frac{\text{Distance of maximum travel of pen on stimulation of PRP with agonist}}{\text{Distance of deflection of pen between PRP and PPP samples}} \times 100 \text{ per cent.}$$

The rate of aggregation was calculated thus:

$$\frac{\text{Per cent maximum extent of aggregation}}{\text{Time (min)}} = \text{Per cent min}^{-1}.$$

Preparation of aggregating agents

1. ADP

ADP (501 mg) was dissolved in 10 ml distilled water. From this stock solution, 10 µl was made up to 10 ml with distilled water. This solution contained 10^{-4}M ADP and was divided into aliquots of 0.5 ml and stored at -20°C. A 0.5 ml aliquot was thawed on the day of use, and kept in ice.

2. Collagen

Collagen stock solution contained 1 mg/ml of native collagen type 1 fibrils from equine tendons. The solution was stored at 4°C and was used untreated or diluted 1 in 10 with modified Tyrode's buffer.

Aggregating agents were added to 0.25 or 0.5 ml platelet samples using a microsyringe (Terumo Ltd., Tokyo, Japan) in volumes not exceeding 10 µl.

2.10 Platelet Resuspension

PRP or PRP-liposome samples (between 3-6 ml) were centrifuged in 10 ml plastic centrifuge tubes at 1000 g for 10 min in a MSE bench centrifuge. The supernatant plasma or plasma/liposome mixture was removed and an equivalent volume of modified Tyrode's buffer was added dropwise down the side of the tube. When all the buffer had been added, the platelets were resuspended by gentle agitation using a plastic Pasteur pipette. This procedure ensured complete resuspension of the platelets within 5 min.

2.11 Preparation of Platelet Membrane Fractions

Platelet membrane fractions were prepared using the method described by Wong and Cheung (1979) to obtain a crude membrane fraction. Platelets were pelleted and resuspended in modified Tyrode's buffer three times to remove the plasma and/or liposomes. PRP was centrifuged at 1000 g for 10 min. After each centrifugation the platelet pellet was resuspended

in an equal volume of buffer. The resuspended platelet samples were then pipetted into siliconised 25 ml beakers placed in ice and sonicated for three 20 second periods with 1 min intervals using a Rapidis 150 probe sonicator (Ultrasonics Ltd., U.K.) with power setting 9 and tuning setting 6.

Sonicated platelet samples were then centrifuged at 3500 g for 20 min to sediment unbroken cells. The supernatants were pipetted into 10 ml plastic centrifuge tubes and centrifuged at 100,000 g for 60 min in a Beckman L2-65B ultracentrifuge. The supernatants were removed and the pellets were rinsed twice with buffer containing 50 mM Tris HCl (pH, 9.0). The membrane pellets were resuspended in 4 ml of this buffer and transferred to siliconised 25 ml glass beakers in ice. The samples were sonicated for three 20 second periods with 1 min intervals to disperse the membrane pellets. The resulting membrane suspensions provided the source of phospholipase A_2, and were kept on ice until used.

2.12 Preparation of 1-acyl-2-[1-^{14}C]-oleoylphosphatidylcholine and 1-acyl-2-[1-^{14}C]-arachidonylphosphatidylcholine

The method used for the production of phosphatidylcholine with radio labelled fatty acid esterified at the sn-2 hydroxyl group of the glycerol moiety was carried out essentially as described by Mulder et al. (1965). Red blood cells were obtained from rats as previously described. The blood sample (10 ml) was centrifuged at 100 g for 15 min and the supernatant platelets were removed. The red cells were washed and resuspended 3 times in Krebs-Ringer solution (Krebs and Henseleit, 1932) containing 0.9 g NaCl, 46 mg KCl, 36.6 mg $CaCl_2$, 38 mg $MgSO_4.7H_2O$, 21.1 mg KH_2PO_4, in 109 ml of distilled water. $NaHCO_3$ solution was prepared by dissolving 1.3 g in 100 ml distilled water. 21 ml of this was added to 109 ml of

Krebs-Ringer solution and gassed for 1 hour with 5 per cent CO_2/ 95 per cent O_2 mixture before use.

The concentrated red cell sample (5 ml) was then centrifuged at 20,000 g for 25 min to give a packed pellet of red cells. 0.5 ml of the sample was taken from the bottom of the centrifuge tube where the cells were most concentrated, and this was used in the incubation system to synthesise radiolabelled phosphatidylcholine. The incubation system was set up as follows:

In a 25 ml round bottom flask, 2.5 mg lysolecithin (Sigma, London) and 10 μCi [1-^{14}C]oleic acid (specific activity 56 mCi/mmol) were sonicated in 1.0 ml Krebs-Ringer bicarbonate buffer for a few minutes in a sonicleaner waterbath under nitrogen. The following were added to this suspension. 1 mg Coenzyme A, 15 mg ATP, 1 mg glucose, 400 i.u. penicillin and 400 i.u. streptomycin. The 0.5 ml red cell sample was added, and the mixture was incubated at 37°C overnight in a slowly shaking water bath.

After incubation, the lipids were extracted by the method of Bligh and Dyer (1959). An equal volume of chloroform:methanol (2:1 v/v) was mixed with the sample. The organic solvent phase was removed and the process repeated twice more. The organic solvent extracts were pooled, the solvent was evaporated under N_2 and the lipids were separated by tlc on kieselgel H type 60 plates in a solvent system containing chloroform:methanol:water, 50:40:1 (v/v). The plate was scanned with a Panax **tlc** scanner, and radioactive peaks corresponding to 1-acyl-2-[1-^{14}C]oleoylPC and [1-^{14}C]oleate were identified near the base-line and at the solvent front respectively. The radiolabelled phospholipid was isolated by scraping the silica gel from the plate in the corresponding area into a beaker containing methanol. This was transferred to a small column and washed with 3 volumes of methanol.

The extract was then evaporated to dryness under N_2, and stored in ethanol.

To check the purity of the substrate, a sample was incubated in 1 ml modified Tyrode's buffer with snake venom as a source of phospholipase A_2. A control sample was also incubated under identical conditions but without phospholipase A_2. After incubation for 30 min, the samples were extracted with organic solvents as described above. The samples were subjected to tlc as described above. In the control sample, all the radioactivity was located on and near the baseline, indicating that all the radioactive fatty acid remained esterified to phosphospholipids. In the sample which had been incubated with phospholipase A_2, all the radioactivity was located at the solvent front indicating that all the radio labelled fatty acid esterified to phospholipid had been hydrolysed. 1-acyl-2-[1-^{14}C]arachidonyl PC was prepared in the same way using [1-^{14}C]arachidonic acid (60 mCi/mmol). Phospholipid determination of the radioactive substrate showed that the specific activity was 3.3 mCi/mmol for both substrates.

2.13 Phospholipase A_2 assay

The source of the enzyme was provided either by once resuspended platelets (0.5 ml) or by a crude platelet membrane preparation (1 ml). Fatty acid free albumin was included in all incubation systems at a final concentration of 3.5 mg/ml in resuspended platelet samples, and 0.5 mg/ml in membrane preparations. The Ca^{2+} concentration was 0.2 mM in resuspended platelet samples and 2.0 mM in membrane preparations obtained by the addition of 100 mM $CaCl_2$ solution prior to commencement of the reaction. The reaction was started by the addition of radiolabelled phospholipid (10 nCi, 2.5 µg of phospholipid in 4 µl ethanol). In membrane preparations, 300 µg of membrane protein was used.

The reaction was terminated by the addition of 1 drop of concentrated HCl, followed by lipid extraction with an equal volume of chloroform: methanol (2:1 v/v) as previously described. Three extractions were carried out, and the organic solvent extracts were pooled, and dried under N_2. The lipid extracts were redissolved in 100-200 µl chloroform, and phospholipids and fatty acids were separated by tlc in a solvent system consisting of chloroform:methanol:water (50:40:1, v/v) as described above. Radioactive peaks were identified using a Panax tlc scanner, and were scraped into vials and counted as described above. Recovery of substrate was approximately 80 per cent.

Phospholipase A_2 activity was assessed to give per cent conversion of substrate to product by the formula:

$$100 \times \frac{\text{cpm of radiolabelled fatty acid peak}}{\text{cpm of radiolabelled phospholipid peak} + \text{cpm of radiolabelled fatty acid peak.}}$$

2.14 Preparation of Indomethacin Solution

Indomethacin (7.16 mg) provided by ICI Pharmaceuticals Division, was dissolved in 5 ml distilled water. This solution was mixed with 5 ml of 2.5 mg/ml Na_2CO_3 solution. This mixture was stirred for 10 min, and another 10 ml of distilled water was added. The concentration of indomethacin in this solution was 1 mM. 10 µl of this was added to 1 ml of resuspended platelets assayed for phospholipase A_2 activity in which [1-^{14}C]arachidonyl PC was used to give a final concentration of 10 µM indomethacin.

2.15 Thromboxane A_2 Generation for Thromboxane A_2 Bioassay

Thromboxane A_2 was generated by aggregating aliquots of rabbit PRP with sodium arachidonate (3×10^{-4} M) in an aggregometer. When irreversible aggregation had occurred (90 sec) samples of plasma containing thromboxane A_2 were rapidly transferred for bioassay.

2.16 Bioassay for Thromboxane A_2

This bioassay was carried out by Dr. M. Johnson while at ICI Pharmaceuticals Division. The thoracic aorta was excised and spirally cut from a healthy rabbit fed a normal stock diet. The tissue was suspended in a 5 ml bath containing oxygenated (95 per cent O_2: 5 per cent CO_2) Krebs-Henseleit solution maintained at 37°C. Contractions of the aorta were recorded isotonically under a resting tension of 1 g. The following antagonists (10^{-7}M) were added to the bathing solution: mepyramine, propranolol, atropine methyl azide in order to ensure that contractions occurred as a result of thromboxane A_2 and not catecholamines or serotonin released during irreversible aggregation. Indomethacin (10^{-5}M) was added throughout. A standard curve comparing contraction with thromboxane B_2 production (measured by radioimmunoassay) was established using normal rabbit PRP. Height of contraction was measured after addition of 50 µl and 100 µl of plasma from each test sample. Thromboxane B_2 levels calculated from the standard curve were taken to be equal to the levels of thromboxane A_2 produced since all A_2 is converted to B_2, and contraction was due solely to thromboxane A_2.

2.17 Materials

All common reagents were purchased from BDH, Sigma or Fisons and were of Analar grade.

Syringes and needles were obtained from Becton, Dickinson and Co., New Jersey, U.S.A.

Plastic blood samples tubes and tubing were obtained from Sterilin Ltd., Teddington, England.

All radioactive chemicals were purchased from The Radiochemical Centre, Amersham, England.

Collagen was obtained from Hormon-Chemie, Munich, FRG.

Cholesterol oxidase, ammonium molybdate and phenol were purchased from BDH Chemicals, Poole, England.

PPO and POPOP were obtained from Koch-Light, England.

ADP L-α-dipalmitoylphosphatidylcholine, (DPPC), and cholesterol were purchased from Sigma (London).

Kieselgel H and kieselgel 60 were obtained from Merck, Sharp and Dohme.

Phospholipase A_2 was purchased from Boehringer GmbH, FRG.

Indomethacin was supplied by ICI Pharmaceuticals Division, Cheshire, England.

Cholesterol-analogues were synthesized by Dr. I.F. Craig and Dr. K.E. Suckling in this laboratory.

Chapter 3

In Vitro Alteration of Rat Platelet Cholesterol Content

3.1 Introduction

Recent studies have shown that human platelets enriched with cholesterol exhibit increased sensitivity to aggregating agents. Conversely, when cholesterol is removed from platelets, the sensitivity to aggregating agents is reduced when compared to control platelets (Shattil et al., 1975). In order to investigate further the role of cholesterol in platelet function, a method for altering the platelet cholesterol content was required which fulfilled certain criteria.

1. Cholesterol enrichment or depletion should be achieved without affecting other platelet components which may be involved in platelet function.

2. The method should involve the minimum of manipulation so that deterioration in the non physiological environment is minimised.

3. The platelets with altered cholesterol content must be easily isolated from the medium at the end of the experiment so that they can be analysed for cholesterol content. It is necessary to remove cholesterol not associated with the platelet membrane prior to analysis, and this includes cholesterol not taken up and cholesterol which has been removed from platelets.

Cooper et al. (1975) described a method for preparing cholesterol-poor, cholesterol-normal and cholesterol-rich phospholipid dispersions. Cholesterol and DPPC were mixed in various proportions and sonicated in buffer to give liposome suspensions of various cholesterol: phospholipid molar ratios (see Chapter 2). These media were used to alter the cholesterol content of red blood cells (Cooper et al., 1975) and of platelets (Shattil et al., 1975). These workers suggested that the molar ratio of cholesterol to phospholipid in the plasma was correlated with the cholesterol content of the red blood cells and platelets. This method was adapted in the present study to prepare

liposomes of various proportions of cholesterol and phospholipid. This chapter describes experiments carried out to test the suitability of this method to alter platelet cholesterol content.

3.2 Preparation of Phospholipid Liposomes for Incubation with PRP

Four types of liposome preparations were used as follows:

1. Cholesterol-poor liposomes. These contained phospholipid only and were used to deplete platelets of cholesterol.

2. Cholesterol-normal liposomes were composed of cholesterol and phospholipid in a molar proportion of approximately 0.6. This was the ratio found by the author in normal rat platelets. This medium was used to maintain the cholesterol content at its endogenous level.

3. Cholesterol-rich liposomes were composed of cholesterol and phospholipid in a molar proportion between 1.0 and 1.5 and were used to enrich platelets with cholesterol.

4. Cholesterol-analogue-rich liposomes were also prepared in order to attempt to load platelets with these cholesterol-like molecules.

The same method was used for preparation of all liposome suspensions. The sterol and phospholipid were mixed in chloroform solution in a round bottom glass flask. The mixture was warmed and the bulk of the solvent was removed using a rotary evaporator to leave a thin film of the lipid mixture spread on the surface of the flask. This was then placed in a vacuum dessicator to remove all traces of solvent from the film. Modified Tyrode's buffer was added to the film at 50^{o}C, a temperature well above the transition temperature for the DPPC, so that the lipid was in a fluid state. This allowed the lipid to form multilamellar dispersions in the aqueous environment. The flask was shaken under a stream of hot water for 3 min to ensure complete removal of the lipid film from the glass. The suspension of lipid was sealed under nitrogen and sonicated at 55-60^{o}C for 3 hours.

At the end of this period, the lipid suspension was centrifuged to remove undispersed lipid, and centrifuged again to ensure no further sedimentation from the liposome suspension. Suspensions containing high proportions of sterol to phospholipid gave rise to sediments of undispersed lipid which were larger than sediments obtained from centrifugation of cholesterol-normal or cholesterol-poor suspensions.

Table 3.1 shows the results of analysis of typical liposome preparations used. It can be seen that there was a greater loss of lipid from cholesterol-rich suspensions than from normal or cholesterol-poor suspensions. In cholesterol analogue rich suspensions, only the C_{26}-analogue suspension showed a significantly greater loss of lipid compared to the cholesterol-normal or cholesterol-poor suspension. It is evident from these results that it is difficult to disperse samples which contain high molar ratios of cholesterol or C_{26}-analogue to phospholipid under these conditions.

Liposomes prepared using DPPC were used in all experiments described for two reasons. Firstly, egg PC was considered liable to oxidation of constituent unsaturated fatty acids during preparation which may have resulted in the liposomes having different properties. Secondly the fatty acid composition of PC would be heterogeneous, and any fatty acids released may affect platelet function (Haslam, 1964).

At the end of the liposome preparation, glucose and albumin were added to final concentrations of glucose (5 mM) and albumin (3.5 mg/ml). Glucose was added to the liposome suspensions at a physiological concentration to maintain platelet function, and albumin was added to stabilize the liposomes (Shattil et al., 1975). All liposome preparations were optically clear. PRP samples were mixed with equal volumes of liposome preparation, and incubated at 37°C. The samples were gently inverted once every half-hour to ensure thorough mixing.

Table 3.1. Composition of lipid mixture at the start, and of the liposome suspension at the end of the preparation method

Liposome Preparation	Starting sterol: phospholipid molar ratio	Final sterol: phospholipid molar ratio	Final phospholipid content (mg/ml)
Cholesterol-poor	0	0	3.02
Cholesterol-normal	0.615	0.65	2.58
Cholesterol-rich	2.2	1.31	1.00
C_{21}-analogue-rich	2.0	0.81	3.15
C_{24}-analogue-rich	2.0	0.88	3.07
C_{26}-analogue-rich	2.0	1.16	1.53

3.3 Analysis of Platelets

Platelets were analysed for total unesterified cholesterol content, total phospholipid content and total protein content, as described in Chapter 2. The amounts of cholesterol and phospholipid were expressed as ratios of weight of each lipid to weight of protein (Shattil et al., 1975). The phospholipid to protein weight ratio was determined in platelets before and after incubation with liposome suspensions to confirm that no phospholipid had been taken up by platelets from the liposomes. The molar ratio of cholesterol to phospholipid was calculated. Since these two lipids are predominantly found in membrane structures, determination of the molar ratio in this way reflects changes in the lipid composition of the platelet membrane.

Platelets were prepared for analysis by centrifugation and resuspending the resultant platelet pellet in platelet washing buffer (see Chapter 2). The supernatant containing plasma and liposomes was removed. This washing process was repeated three times to ensure removal of all the liposomal lipids. The buffer used for washing the platelets contained no inorganic phosphates. This solution differs therefore from buffers used previously (Gaintner et al., 1962). The use of a phosphate free buffer precludes the possibility of phosphates being extracted with organic solvents which would give false results for platelet phospholipid content.

3.4 Viability of Method for Platelet Cholesterol Content Alteration

1. Effect of liposomes on platelet composition

Table 3.2 shows results from an experiment to observe the effect of liposome suspensions of different lipid content on platelet composition. A concentrated cholesterol-normal liposome suspension was prepared so that by serial dilution, liposome suspensions of decreasing lipid content could be obtained. The cholesterol-normal liposome suspension

Table 3.2. Analysis of rat platelets incubated with cholesterol-normal liposome suspensions

Final Phospholipid Concentration (mg/ml)	Cholesterol: phospholipid molar ratio	Cholesterol: protein weight ratio	Phospholipid: protein weight ratio
3.60	0.570	0.052	0.185
2.16	0.580	0.055	0.195
0.72	0.625	0.059	0.186
0.00	0.533	0.054	0.205

Rat PRP samples were incubated for 3 hrs at 37°C with liposome suspensions prepared with different lipid concentrations.

was prepared as described previously, except that lipids were sonicated in 6 ml instead of 10 ml of buffer. Table 3.2 shows the four dilutions made from this preparation with the final phospholipid concentration of each. The range of concentrations of liposomal phospholipid prepared was wider than was found with the liposome suspensions used in experiments carried out in this project.

Table 3.1 shows the results of analysis of platelets after 3 hours incubation with an equal volume of liposome suspension. The molar ratio of cholesterol to phospholipid, and the weight ratio of cholesterol to protein is essentially the same in all samples except for the sample incubated with Tyrode's buffer alone. These results were obtained from single tests only, but it is considered that the experimental error is of the order of 5 and no more than 10 per cent (see Discussion).

2. Phospholipid uptake by platelets from liposomes

The results in Table 3.1 indicate that platelets incubated with liposomes make no net gain of phospholipid. The possibility of phospholipid exchange between platelets and liposomes was therefore examined. Cholesterol-normal liposomes were prepared in the usual way with the addition to the lipid mixture of 150 nCi of 1-acyl-2-[1-^{14}C]oleoyl PC prior to sonication in buffer. This preparation was incubated with PRP for 3 hours. At intervals, samples of platelets were isolated and the lipids extracted. Total radioactivity in each sample was determined and the results expressed in Table 3.3 give the percentage of the total radioactivity in an equivalent volume of incubation mixture that was detected in the platelet extracts. Two or three washes of isolated platelets were carried out to give an indication of the efficiency of the washing procedure at removing liposomes. The results in Table 3.3 indicate very little phospholipid

Table 3.3. Uptake of phospholipid from liposomes by rat platelets

Incubation time (min)	10	60	180
% radioactivity found in platelets	0.64, 0.63	1.73, 1.56	0.60, 1.10
Number of washes of platelet sample	3	2	3

Rat PRP (5 ml) was incubated with a cholesterol-normal liposome suspension (5 ml) at 37°C with ½ hourly inversion. This liposome suspension contained a total of 0.15 μCi [1-^{14}C] oleoyl PC (specific activity 3.3 μCi/mmol). 1 ml PRP-liposome samples were prepared for analysis of uptake of radiolabelled phospholipid by centrifuging and resuspending either 2 or 3 times as shown. Radioactivity was determined in the platelet sample and calculated as % of total radioactivity in 1 ml PRP-liposome mixture.

exchange occurred during the 3 hours, and that the washing procedure was efficient for the removal of the liposomes.

3. Cholesterol uptake from cholesterol-rich liposomes

Figures 3.1-3.3 summarize the results from analysis of platelets mixed with liposome suspensions to either 1) maintain or 2) increase the cholesterol:phospholipid molar ratio during a time course of 5 hours.

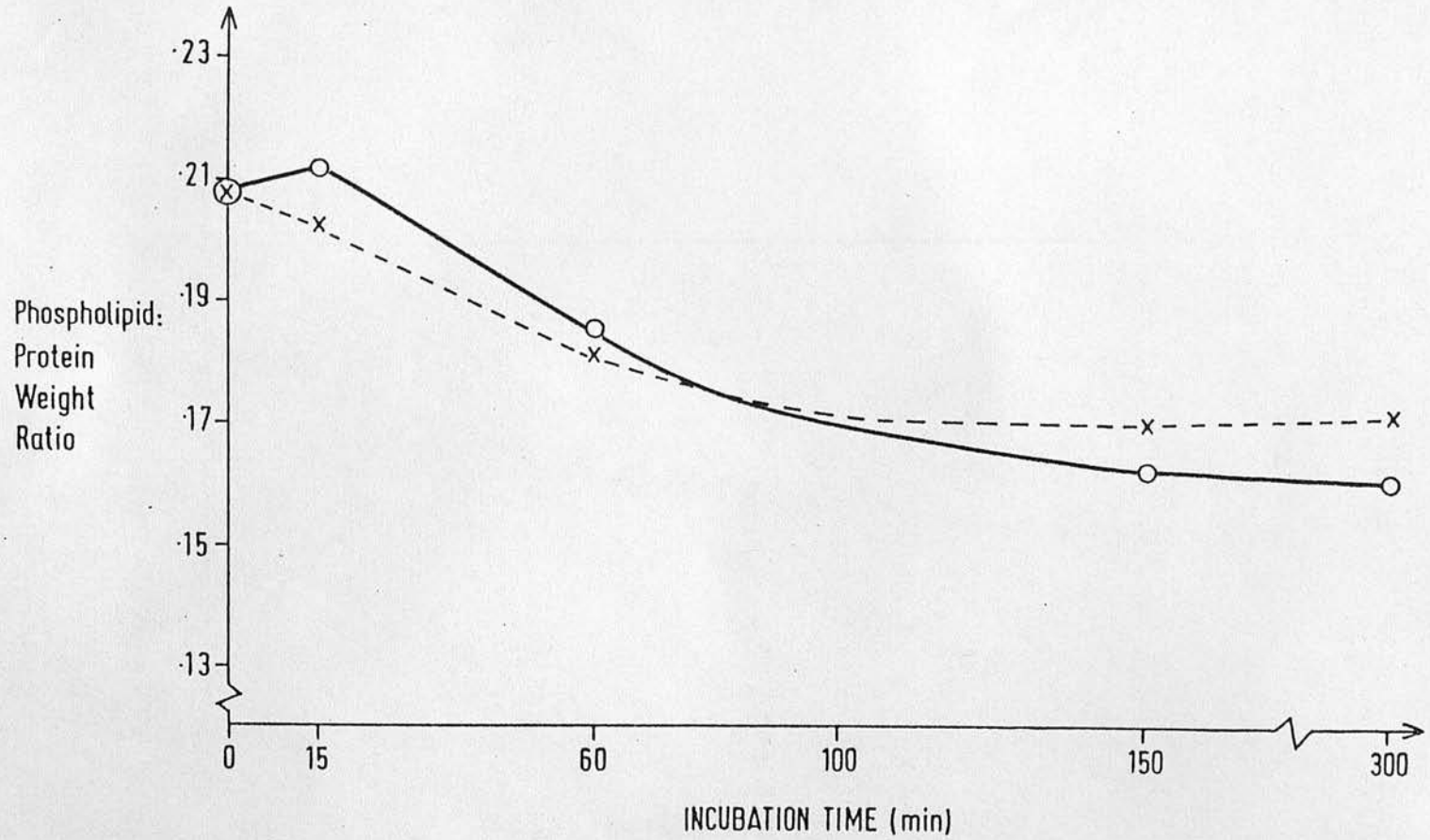
Figure 3.2 shows that the cholesterol:protein weight ratio was increased in the cholesterol-rich platelets. This would be expected if there was a net transfer of cholesterol from the liposomes to the platelets. In the control platelets, the ratio was seen to fall slightly to a steady value after 3 hours. This observation suggests that either cholesterol was being lost, or that protein was being taken up by the platelets during the incubation period. Figure 3.1 shows a comparison of the phospholipid:protein weight ratios in the two samples. This ratio also decreased with time to a steady level after 3 hours. Phospholipid was either being lost, or protein was being taken up by the platelets, but this effect was the same in both samples. Figure 3.3 however shows that the cholesterol:phospholipid molar ratio remained unchanged in the control sample, whereas in the cholesterol enriching system, the ratio was increased after 3 hours by 40 per cent.

These results suggest that cholesterol enrichment could be achieved by this method, but incubation of the platelets induced either protein uptake, or a loss of equimolar amounts of cholesterol and phospholipid. The results in Table 3.2 suggest that the concentration of liposomal lipid in the incubation system does not cause this effect, since ratios are the same in platelets incubated without liposomes, and with widely different amounts of liposomal lipid. These composition changes in platelets were probably due to deterioration of the cells outside the

normal physiological environment. Shattil et al. (1975) observed no significant changes in lipid:protein weight ratios in human platelets treated in this way. It is possible that rat platelets were more susceptible to changes in composition under these conditions. Hamid et al. (1980) showed that in human platelet concentrates stored at 20°C for 72 hours, there was a 15 per cent loss of total cholesterol, and a 7-11 per cent loss of total phospholipid. The loss of lipids shown by Hamid et al. (1980) may be occurring by a mechanism similar to the composition changes observed in the present study with rat platelets. The process in rat platelets appeared to be more rapid, but this was possibly due to the temperature being 37°C as opposed to 20°C used by Hamid et al. (1980). The reason for the composition changes remains obscure, but it is important to emphasize that this process did not influence or affect platelet function, and was not due to the presence of liposomes.

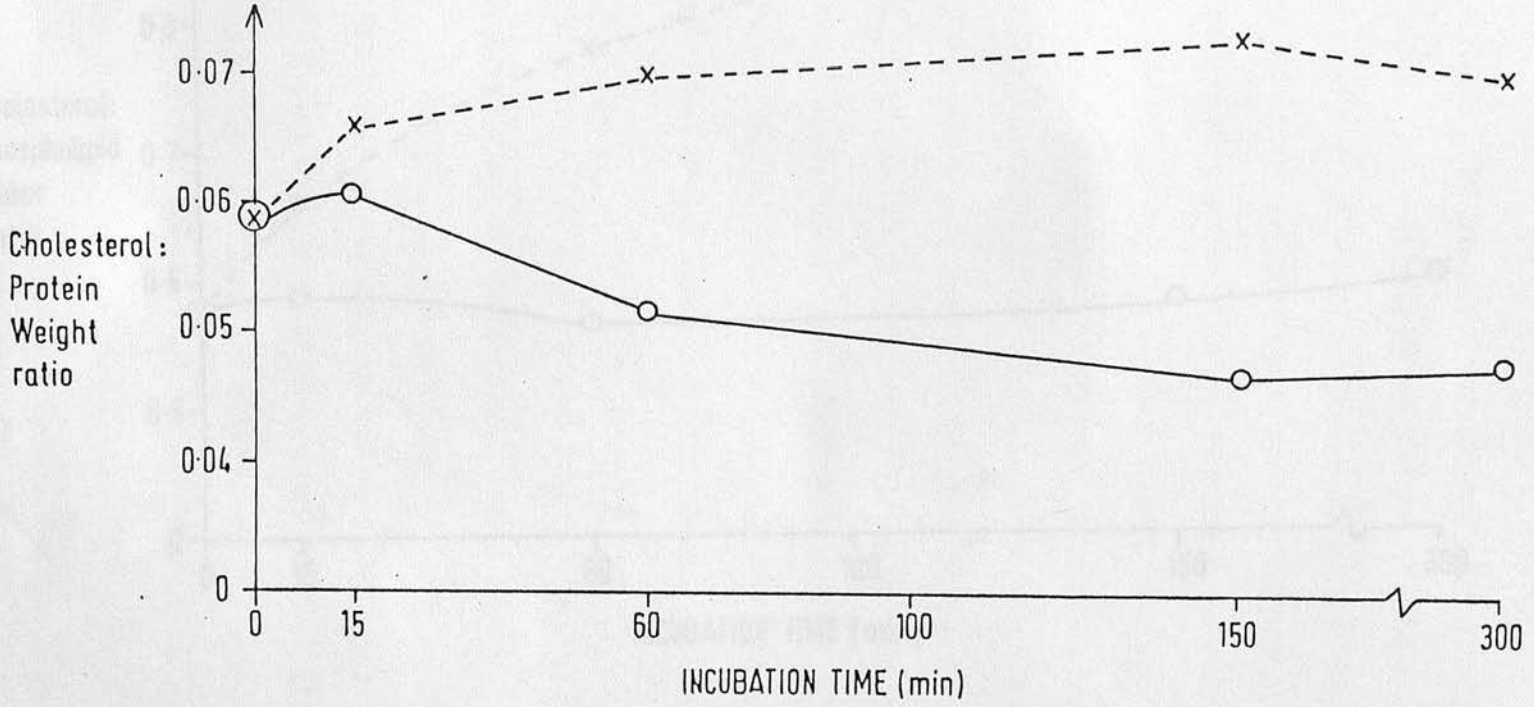
The results shown in figures 3.1-3.3 were obtained from one experiment. It has consistently been found however that incubation of rat and human platelets for three hours with cholesterol rich liposomes leads to an increase in the level of cholesterol in these platelets. Table 5.1 of Chapter 5 shows analysis of rat platelets obtained from the six experiments reported in that chapter. The cholesterol:phospholipid molar ratio was increased by an average of 20 per cent. Table 7.1 of Chapter 7 shows that human platelets also showed a significant increase in cholesterol content after in vitro enrichment. Table 7.1 also shows that the cholesterol content of human platelets was significantly reduced on incubation with cholesterol-poor liposomes. Finally table 8.7 shows that incubation of rat platelets with cholesterol rich liposomes resulted in an increase in the cholesterol content of crude membrane fractions prepared from these platelets.

Figure 3.1. Phospholipid:protein weight ratio of whole rat platelets



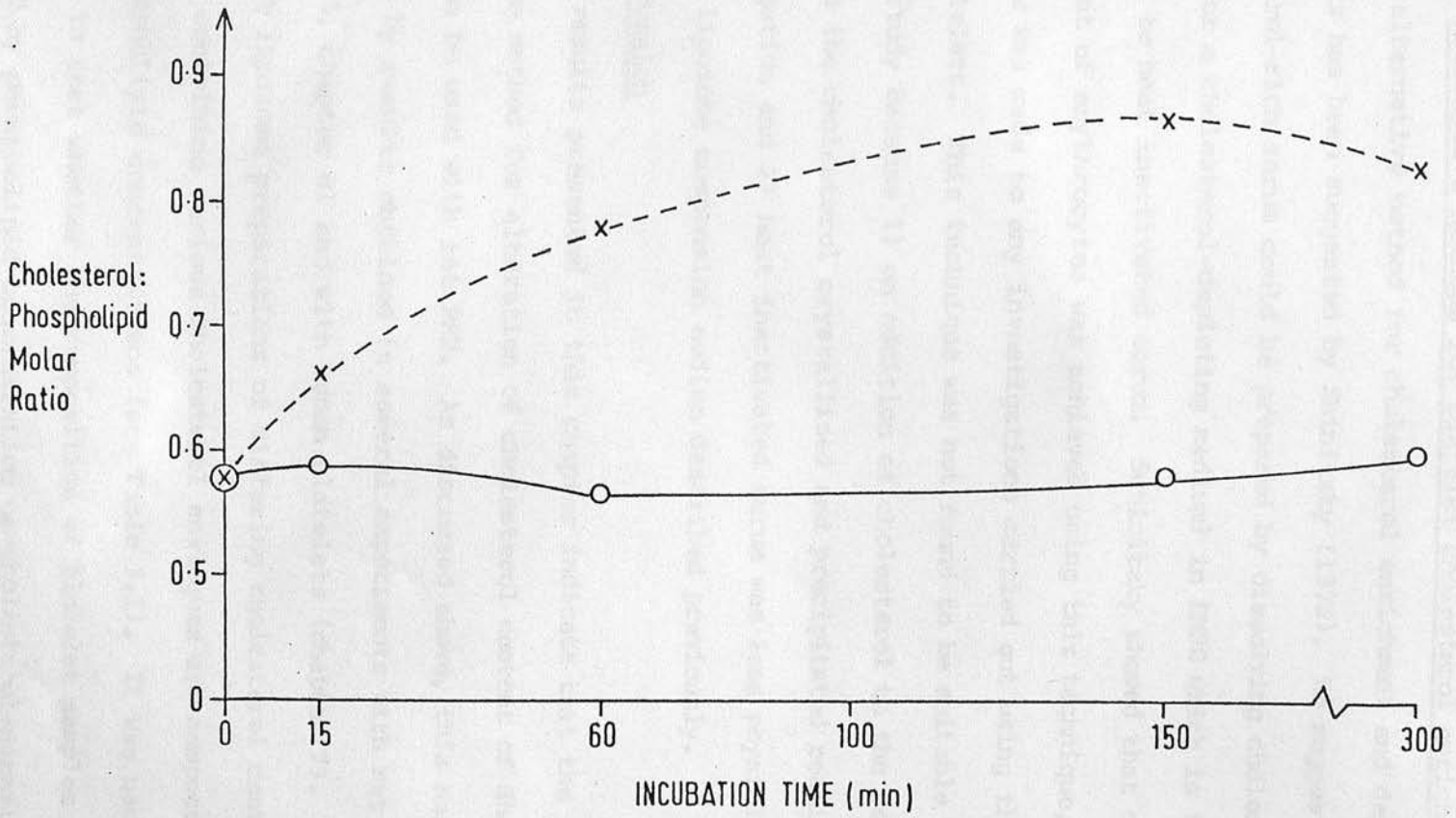
PRP samples were incubated with cholesterol-normal liposomes (o——o) and cholesterol-rich liposomes (x---x) at 37°C and inverted ½ hourly.

Figure 3.2. Cholesterol:protein weight ratio of whole rat platelets



PRP samples were incubated with cholesterol-normal liposomes (o——o) and cholesterol-rich liposomes (x---x) at 37°C and inverted ½ hourly.

Figure 3.3. Cholesterol:phospholipid molar ratio of whole rat platelets



PRP samples were incubated with cholesterol-normal liposomes (o——o) and cholesterol-rich liposomes (x---x) at 37°C and inverted ½ hourly.

In view of the results outlined above the present studies showed that incubation of rat or human platelets with phospholipid liposomes of differing cholesterol composition reproducibly altered platelet cholesterol content.

4. Alternative methods for platelet cholesterol enrichment

An alternative method for cholesterol enrichment and depletion of platelets has been suggested by Shinitzky (1978). He suggested that a cholesterol-rich serum could be prepared by dissolving cholesterol (or PC for a cholesterol-depleting medium) in DMSO which is then added directly to heat inactivated serum. Schinitzky showed that cholesterol enrichment of erythrocytes was achieved using this technique, but no reference was made to any investigations carried out using this method with platelets. This technique was not found to be suitable in the present study because 1) on addition of cholesterol to the serum as described the cholesterol crystallised and precipitated readily on centrifugation and 2) heat inactivated serum was less physiological than the liposome suspension medium described previously.

3.5 Discussion

The results presented in this chapter indicate that the liposome incubation method for alteration of cholesterol content of Shattil et al. (1975) can be used with rat PRP. As discussed above, this has been confirmed by results obtained in several experiments with rat platelets (chapter 5, chapter 8) and with human platelets (chapter 7). It was found that liposome preparations of differing cholesterol content and liposomes containing various cholesterol analogues are composed of final phospholipid concentrations (see Table 3.1). It was necessary therefore to test whether the composition of platelet samples was influenced by phospholipid concentration of cholesterol-normal liposomes with which they were incubated. Table 3.2 shows analyses of single

samples of rat platelets incubated with cholesterol normal liposomes of different final phospholipid concentration. Statistical analysis was therefore not possible but the range of error for any single result was found to be normally $\pm$ 5 per cent, but certainly no more than $\pm$ 10 per cent. Allowing for an error within this range, the results indicate that no difference in platelet composition occurred as a result of different phospholipid concentrations. However, the platelet sample incubated with Tyrode's buffer alone showed a low cholesterol content, although in subsequent experiments where rat PRP samples have been incubated with Tyrode's buffer alone, analysis shows that the cholesterol content of these platelets is most probably not significantly different from platelets incubated with cholesterol-normal liposomes (see tables 8.3, 8.4, chapter 8).

It was also important to test whether phospholipid uptake by platelets from liposomes occurred. This would be an undesirable event, one problem would be interpretation of analysis data if part of the phospholipid measured was derived from liposomes. The results of table 3.3 indicate that very little liposome phospholipid was taken up by platelets, even after three hours incubation, showing that reliable platelet analysis could be carried out.

The technique of alteration of platelet cholesterol content by the liposome incubation method has been used in all the present studies. Precautions have been taken however to ensure the reliability of all analysis results. In each experiment a control sample of PRP incubated with cholesterol-normal liposomes was included and treated in the same way as the other samples. Also, analysis for platelet protein, cholesterol and phospholipid was carried out on all samples to confirm that platelets did not take up liposome phospholipid.

This method of cholesterol content alteration has been used with

uman platelets (chapter 7) and rat platelets (chapter 8). In addition arious cholesterol analogues have been incorporated into rat platelets y this method (chapter 8). It has been possible to investigate platelets nriched with cholesterol in aggregation studies and in assays for phospho- ipase A_2 activity. The technique used to assay for phospholipase A_2 ctivity is described in the following chapter.

Chapter 4

Phospholipase A_2 Assay Methods

4.1 Introduction

It is now generally accepted that platelets synthesize thromboxane A_2 when stimulated by various aggregating agents. It has been suggested that the thromboxane A_2 formed induces irreversible aggregation and secretion (Blackwell et al., 1977; Hamberg et al., 1975). Inhibition of thromboxane A_2 synthesis from PGH_2 was suggested by Gorman et al. (1977a) to be responsible for the inhibition of the second wave of aggregation induced by ADP or adrenaline observed in human platelets. As was discussed in Chapter 1, it may be that the synthesis of PGH_2 is more important than thromboxane A_2 in mediating platelet aggregation and secretion (Westwick et al., 1981). However, it is clear that metabolism of arachidonic acid is very important (Marcus, 1978). Very little free arachidonic acid is present in unstimulated platelets (Marcus, 1969) and a prerequisite for the conversion of arachidonic acid to prostaglandins is that the precursor is in the free acid form (Lands and Samuelsson, 1968).

Phospholipase A_2 in platelets hydrolyses the sn-2 ester bond between the fatty acid carboxyl group and the 2nd carbon atom of the glycerol moiety of the phospholipid (Jesse and Franson, 1979). The enzyme is membrane bound (Lapetina et al., 1978) and when activated releases fatty acids from membrane phospholipids (Kannagi and Koizumi, 1979). It was shown by Bills et al. (1976) and Blackwell et al. (1977) that platelets prelabelled with [1-^{14}C] arachidonic acid released this fatty acid from membrane phospholipids when stimulated with either collagen or thrombin. This reaction catalysed by phospholipase A_2 could therefore be an important rate limiting step in the supply of free arachidonic acid (Wong and Cheung, 1979; Blackwell et al., 1977).

Cholesterol-enriched human platelets showed increased sensitivity to aggregating agents compared to control or cholesterol-depleted

platelets (Shattil et al., 1975) and also showed increased thromboxane B_2 production compared to cholesterol-depleted platelets (Stuart et al., 1980a). In the study by Stuart et al. it was possible that the enhanced thromboxane B_2 production in cholesterol-enriched platelets was a result of enhanced arachidonic acid release from membrane phospholipids by phospholipase A_2. In the present project, it was therefore of interest to investigate further the possible influence of cholesterol on phospholipase A_2 activity. In order to investigate the possible effect of the important membrane component cholesterol on the membrane bound enzyme, methods of assay of phospholipase A_2 activity were investigated in the present work.

4.2 Approaches to Phospholipase A_2 Assay

An important consideration for an assay method for a membrane bound enzyme is the problem of delivering the substrate to the enzyme. In the case of phospholipase A_2, the lipid environment in which it is situated provides the substrate. Arachidonic acid is released from the membrane and metabolised further. The other product, the corresponding lysophospholipid, is presumed to be reacylated very rapidly (Billah et al., 1980). Two principal methods of assaying phospholipase A_2 activity have been investigated. In the first, the method of prelabelling platelet phospholipids with radiolabelled fatty acid, and studying the extent of release of this fatty acid on stimulation of the platelets was investigated. In the second, the method involved presenting a phospholipid substrate with radiolabelled fatty acid esterified at the sn-2 carbon atom to either resuspended platelets or a crude platelet membrane fraction.

4.3 Prelabelling Rat Platelets with Radiolabelled Fatty Acid

The method adopted for incorporation of radiolabelled fatty acid into platelet phospholipids was that described by Bills et al. (1976).

Tables 4.1 and 4.2 show results for the incorporation of [1-^{14}C]oleic acid (specific activity 56 mCi/mmol) and [1-^{14}C] arachidonic acid (specific activity 60 mCi/mmol). The incorporation of [1-^{14}C] oleic acid was investigated because oleic acid released by the action of phospholipase A_2 would not be subject to further metabolism by enzymes involved in arachidonic acid metabolism. Further metabolites of arachidonic acid such as PGH_2 and thromboxane A_2, potent pro-aggregating agents, may influence the activity of phospholipase A_2. It was anticipated that an inhibitor would be required to prevent further metabolism of arachidonic acid so that the arachidonic acid released could be isolated by tlc as described in Chapter 2.

The results in Table 4.1 show that of the total radiolabelled oleic acid added to the platelet rich mixture, only 0.8 per cent was incorporated after incubation at 37°C for one hour. The incorporation was increased to 2.2 per cent when once resuspended platelets (in buffer) were incubated in the same way. As Table 4.1 shows, when lipids were extracted from platelets incubated with [1-^{14}C] oleate, much of the radiolabel remained unincorporated and in the free acid form. Of the total radiolabelled oleate added, 3.3 per cent was recovered as unincorporated fatty acid when incubated in PRP, and 14 per cent when incubated with resuspended platelets. The results show that even after washing the platelets, a significant amount of fatty acid remains associated with platelets which has not been incorporated.

Table 4.2 shows the results for incorporation of [1-^{14}C] arachidonic acid incubated in PRP which was carried out under the same conditions as for [1-^{14}C] oleic acid incorporation. The results show that the 7.4 per cent of the [1-^{14}C] arachidonic acid originally added was incorporated into platelet phospholipids. This indicates that arachidonic acid may be more readily incorporated than oleic acid into platelet

Table 4.1. [1-^{14}C] Oleic acid incorporation into rat platelets

	[1-^{14}C] oleate incubated with PRP	[1-^{14}C] oleate incubated with once resuspended platelets
[1-^{14}C] oleate incorporated into platelet phospholipids (%)	0.6, 1.0	2.0, 2.4
[1-^{14}C] oleate recovered as free fatty acid (unincorporated) (%)	3.2, 3.4	12.9, 15.1

5 ml rat PRP (left column) or 5 ml rat platelets resuspended in modified Tyrode's buffer (right column) were incubated at 37°C for 60 min with 0.1 μCi [1-^{14}C] oleate (56 mCi/mmol) in ethanol (20 μl). Platelet samples (1 ml) were washed 3 times and analysed for ^{14}C content. Duplicate tests were carried out on each sample. Results show the percentage of the total radiolabel added located in the platelets.

Table 4.2. [1-^{14}C] Arachidonic acid incorporation into and release from rat platelets

	[1-^{14}C] arachidonate incubated with PRP (n=1)	[1-^{14}C] arachidonate labelled resuspended platelets stimulated with 20 µg/ml collagen (n=1)
[1-^{14}C] arachidonate in platelet phospholipid (%)	7.4	7.0
[1-^{14}C] arachidonate recovered as free fatty acid (unincorporated) (%)	18.0	20.0

Rat PRP (10 ml) was incubated with 0.1 µCi [1-^{14}C]arachidonic acid (60 mCi/mmol) added in 20 ul ethanol at 37°C for 60 min. Platelet samples were washed 3 times and resuspended in modified Tyrode's buffer. An aliquot of this platelet sample was analysed for ^{14}C content (left column). The remaining sample was incubated for 10 min with 20 µg/ml collagen, and was then analysed for ^{14}C content (right column).

Results show the proportion of the total radiolabel added located in the platelets and are for single tests only.

phospholipids in general agreement with the results of Bills et al. (1977). However using the technique of fatty acid incorporation and the platelet washing procedure described by Bills et al. (1976) to remove unincorporated fatty acid, it was found in the present study that unincorporated fatty acid remained in the platelets.

As shown in Table 4.2, [1-^{14}C] arachidonic acid was incorporated into rat platelets when incubated with PRP. Of the total added, 7.4 per cent was incorporated into phospholipids, and 18 per cent remained as free fatty acid. It was of interest to see whether any of the incorporated [1-^{14}C] arachidonic acid was released by stimulation of these platelets with collagen. Samples of platelets containing [1-^{14}C] arachidonic acid were stimulated with a concentration of collagen (typically 20 µg/ml) which induces irreversible aggregation. The results in the second column of Table 4.2 show that 10 min incubation of platelet samples with 20 µg/ml of collagen did not induce a significant change in the [1-^{14}C] arachidonic acid content of platelet phospholipids. These findings indicated that this approach was unsuitable for assaying phospholipase A_2 activity in rat platelets.

Compared to the work of Bills et al. (1976, 1977) with human platelets, far less arachidonic acid was incorporated into rat platelet phospholipids in the present study. Human platelets were reported by Bills et al. (1976) to have incorporated between 12 and 40 per cent of the total [1-^{14}C] arachidonic acid added to PRP, and in a subsequent study this figure was 45.9 per cent (Bills et al., 1977).

4.4 Use of Radiolabelled Phospholipid Substrate for Phospholipase A_2 Assay

[1-^{14}C] oleoyl PC has been used previously to assay for platelet membrane phospholipase A_2 activity (Blackwell et al., 1977; Jesse and

Franson, 1979). 1-acyl-2-[1-^{14}C] oleoyl PC was prepared as described in Chapter 2 by the method of Mulder et al. (1965). The positional specificity of the labelled fatty acid was confirmed by incubating a sample with snake venom containing phospholipase A_2. After 30 min, no labelled PC was detected, indicating that all the [1-^{14}C] oleic acid was originally esterified at the sn-2 position of the glycerol moiety. As described in Chapter 2 the percentage conversion of [1-^{14}C] oleoyl PC to [1-^{14}C] oleic acid was determined and used as an index of phospholipase A_2 activity. [1-^{14}C] Arachidonoyl PC was also prepared and used as a substrate in studies discussed in Chapter 6.

4.5 Assay for Phospholipase A_2 in once Resuspended Rat Platelets

Table 4.3 summarizes results of two experiments carried out to investigate the effect of Ca^{2+} concentration and different doses of collagen on phospholipase A_2 activity in once resuspended rat platelets. A PRP sample was centrifuged and the platelet button was resuspended in modified Tyrode's buffer with glucose (5.5 mM) and fatty acid free albumin (2 mg/ml). 0.5 ml samples of resuspended platelets were used for each assay. The samples were incubated at 37^{o}C and stirred at 1000 rpm prior to treatment. [1-^{14}C] Oleoyl PC (10 nCi) was added to the sample, and after 1 min preincubation the collagen was added to the same sample . After incubating and stirring for 10 min the lipids were extracted from the phospholipase A_2 assay sample by the method of Bligh and Dyer (1959) as described in Chapter 2. Phospholipids and fatty acids extracted from this sample were separated by tlc, and the percentage conversion was calculated. The results in Table 4.3 demonstrates a possible requirement of calcium ions for phospholipase A_2 activity in once resuspended rat platelets.

Table 4.4 summarizes the results of another experiment where phospholipase A_2 activity of resuspended platelets was recorded to

Table 4.3 Effect of [Ca^{2+}] and collagen on phospholipase A_2 activity in resuspended rat platelets

		Experiment 1	Experiment 2
Collagen final concentration µg/ml	Ca mM	Phospholipase A_2 activity %	Phospholipase A_2 activity %
0	0	–	2
0	0.2	6.0	2.8
2	0.2	9.0	3.5
10	0	5.0	2.0
10	0.2	15.0	4.3

Platelets were resuspended in modified Tyrode's buffer pH 7.4 glucose (5.5 mM) alubmin (2 mg/ml). Samples (0.5 ml) were incubated at 37°C and stirred at 1000 rpm. Phospholipase A_2 assays were carried out by the addition of [1-^{14}C]oleoyl PC (10 nCi) followed after 1 min by the collagen. The reaction was terminated and lipids extracted as described in Chapter 2 after 10 min incubation.

Table 4.4 Relationship between A_2 activity and collagen concentration in resuspended rat platelets

Collagen final concentration µg/ml	Phospholipase A_2 activity per cent conversion
0	1.7, 1.7
1	1.9, 2.1
4	2.9, 4.3
8	5.3

Results shown are for two tests carried out for all collagen concentrations except for 8 µg/ml collagen, this result being for a single test.

Samples of platelets (0.5 ml) were incubated at 37°C and stirred at 1000 rpm, $[CaCl_2]$ = 0.2 mM. [1-^{14}C]oleoyl PC (10 nCi) was added, and after 1 min, collagen was added. The reaction was terminated and lipids extracted as described in Chapter 2, after 10 min incubation.

different concentrations of collagen. Platelets were resuspended as described above, and $CaCl_2$ (100 mM) was added to give a final concentration of 0.2 mM Ca^{2+}. This concentration of Ca^{2+} could not be exceeded without the induction of spontaneous aggregation of platelets on stirring at 1000 rpm. All other conditions were the same as for the experiments recorded in Table 4.3. The results in Table 4.4 suggested that there was a correlation between collagen concentration and phospholipase A_2 activity.

The results in Tables 4.3 and 4.4 revealed that phospholipase A_2 activity could be assayed in once resuspended rat platelets. This method has therefore been used to compare the phospholipase A_2 activity of normal and cholesterol enriched resuspended platelets from rats (Chapter 5) and rabbits (Chapter 6).

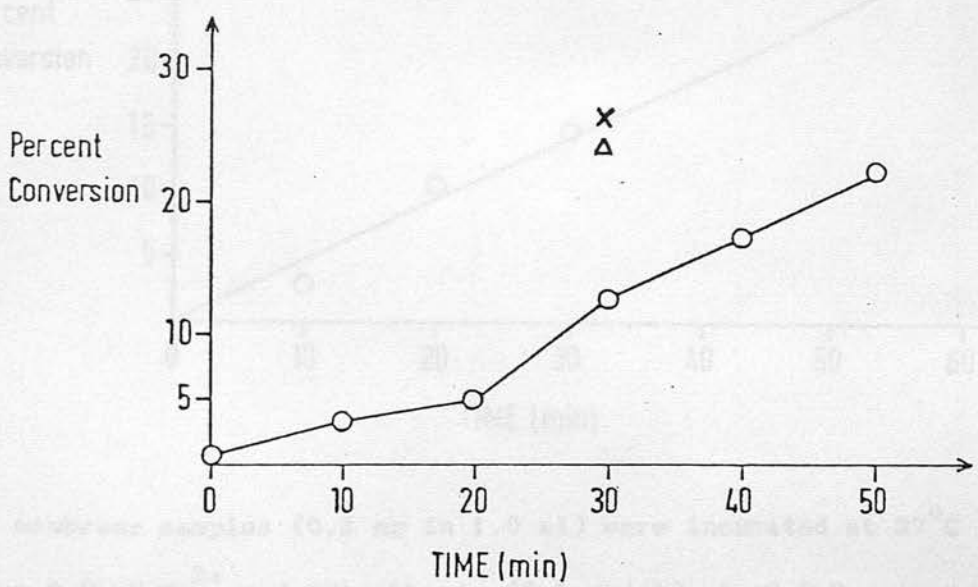
4.6 Assay for Phospholipase A_2 in a Crude Rat Platelet Membrane Preparation

Crude platelet membrane fractions were prepared as described in Chapter 2 following essentially the method of Wong and Cheung (1979). Platelets were resuspended in modified Tyrode's buffer and sonicated to rupture the platelets. The first low speed centrifugation was then carried out to sediment large fragments and unbroken platelets. The supernatant was removed and centrifuged again at high speed to give a pellet of concentrated membranous material. The supernatant from this centrifugation was discarded, and the pellet was rinsed with membrane suspending buffer in order to remove soluble proteins. The membrane pellet was dispersed and resuspended by sonication.

Samples of this suspension were analysed for protein. In this way it was possible to incorporate the same concentration of membrane protein in each sample to be tested for phospholipase A_2 activity. Fatty acid free albumin was added to each of the final reaction mixtures to sequester fatty acid released by hydrolysis of the phospholipid substrate. Lipid extraction was carried out on all samples for determination of phospholipase A_2 activity at the end of the incubation period as described previously. Small aliquots of the crude membrane preparations were set aside for analysis of cholesterol and phospholipid content. Lipids were extracted from samples by the method of Bligh and Dyer (1959) as described in Chapter 2.

Preliminary experiments revealed that phospholipase A_2 activity could be assayed in crude platelet membrane preparations. Figures 4.1-4.4 show results for conversion of [1-^{14}C] oleoyl PC to [1-^{14}C] oleic acid by membrane preparations under various conditions. The five figures represent results obtained from experiments using five different crude membrane preparations. Figure 4.1 shows the conversion of [1-^{14}C] oleoyl PC to [1-^{14}C] oleic acid was time dependent, and single assays with fatty acid free albumin (0.5 and 1.0 mg/ml) showed that this protein enhanced phospholipase A_2 activity in agreement with other reports (see Discussion). Figure 4.2 confirms that phospholipase A_2 activity as measured by this method was time dependent in the presence of 0.5 mg/ml albumin and 2.0 mM Ca^{2+} at pH 9.0. The results in Figures 4.1 and 4.2 were obtained from different membrane preparations. Since there were variations between the phospholipase A_2 activities of different preparations, the results of Figures 4.1 and 4.2 cannot be compared directly with each other. The results in Figure 4.1 suggests that albumin is required for optimal conversion of substrate to product. Figure 4.3 shows the phospholipase A_2 activity is dependent on the concentration of Ca^{2+}. 2.0 mM Ca^{2+} was considered to be a suitable

Figure 4.1. Time dependence of phospholipase A_2 activity in rat platelet membrane fractions



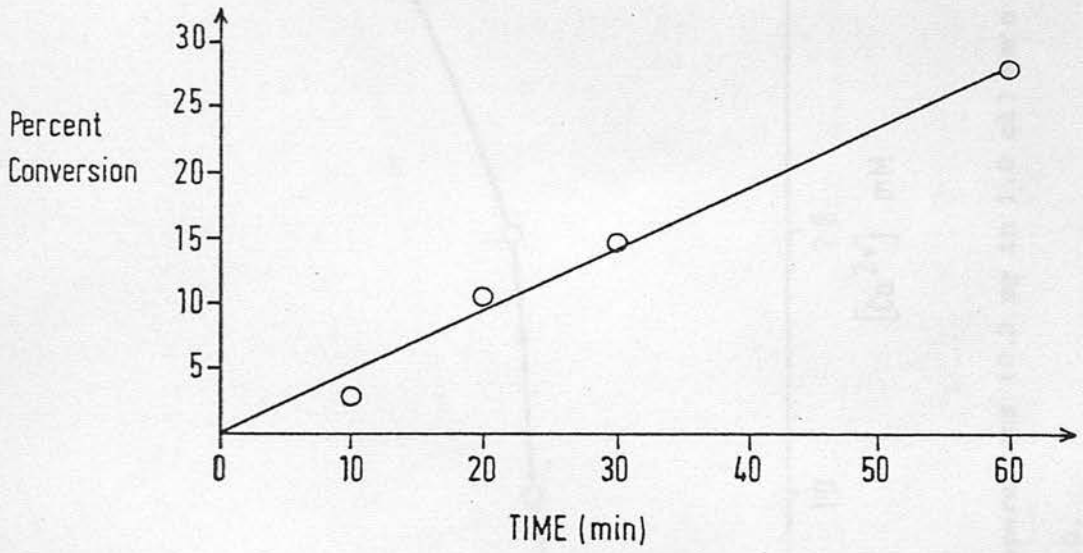
Samples of platelet membrane fractions (0.3 mg in 1.0 ml) were incubated at 37°C with 2.0 mM Ca^{2+} at pH 9.0.

o—o = samples incubated without FFA albumin

x = sample incubated with FFA albumin (0.5 mg/ml)

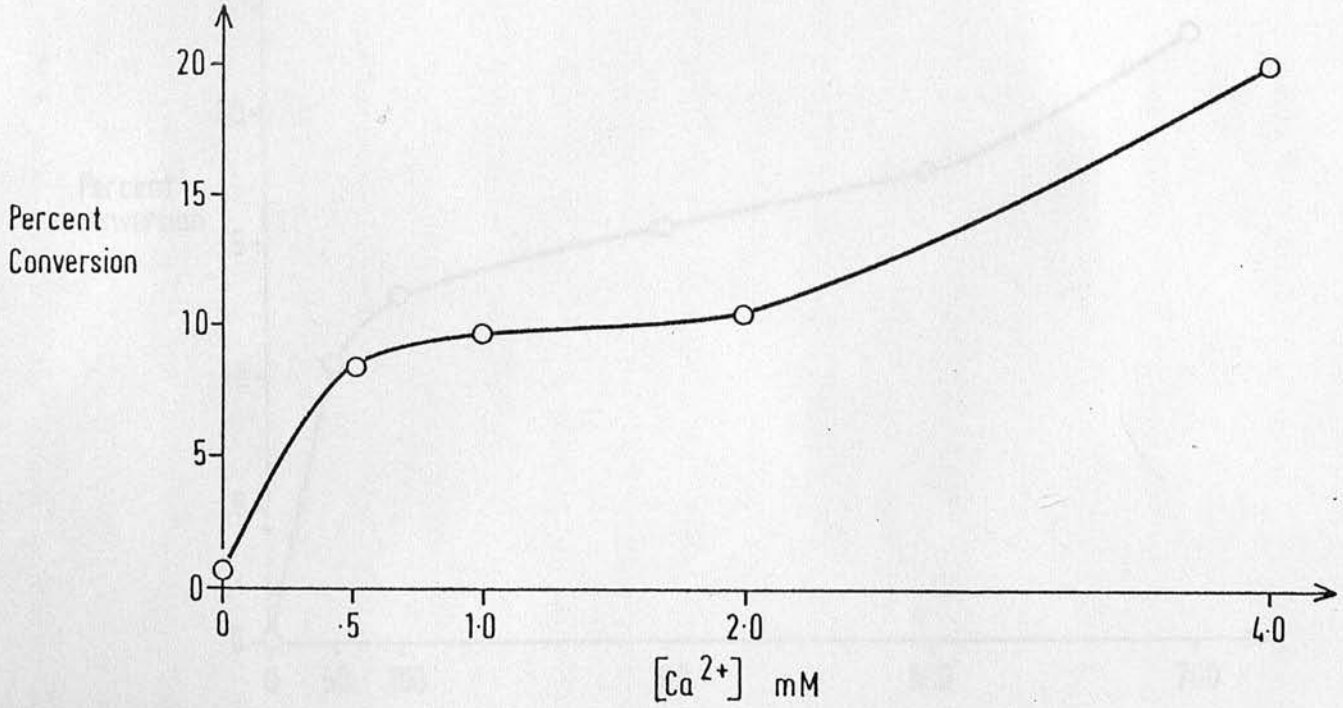
Δ = sample incubated with FFA albumin (1.0 mg/ml)

Figure 4.2. Time dependence of phospholipase A_2 activity in rat platelet membrane fractions in the presence of FFA albumin



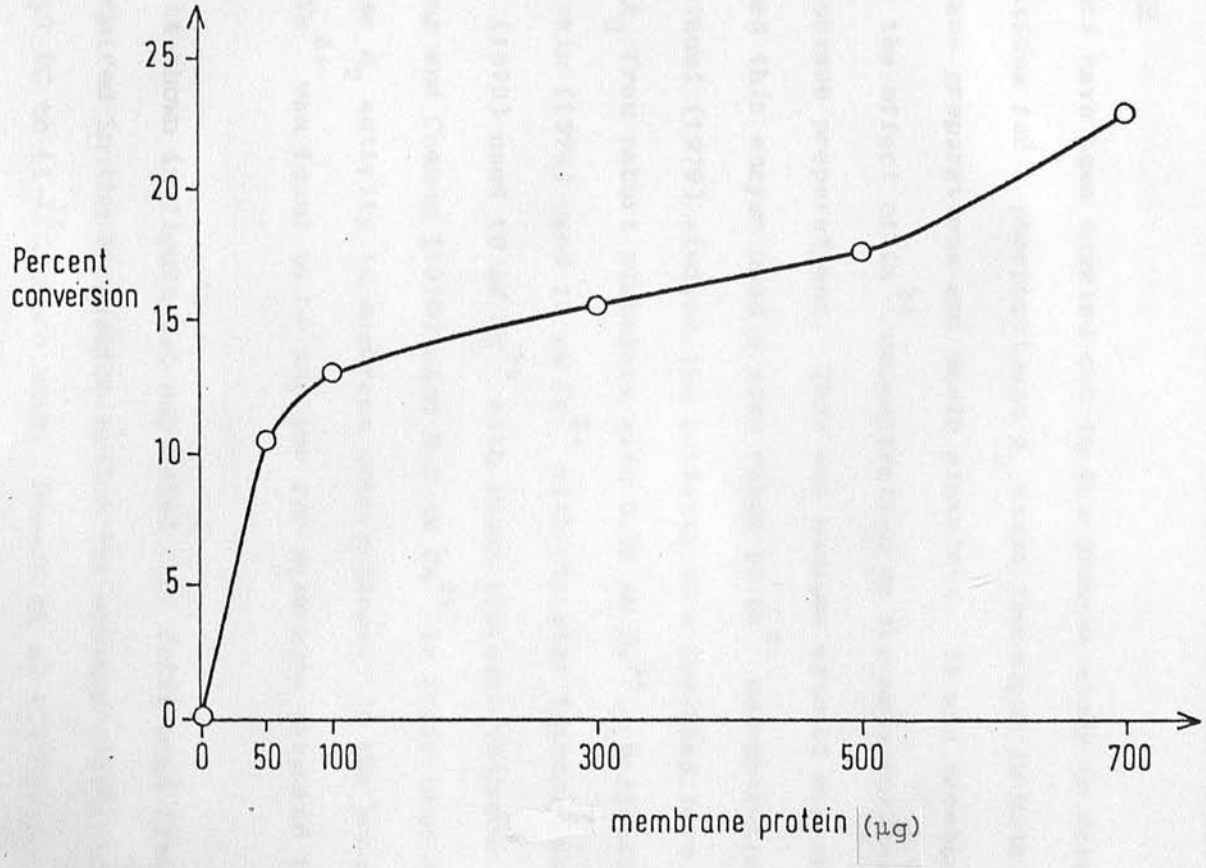
Platelet membrane samples (0.3 mg in 1.0 ml) were incubated at 37°C containing 2.0 mM Ca^{2+} and FFA albumin (0.5 mg/ml) at pH 9.0.

Figure 4.3. Ca^{2+} dependence of phospholipid A_2 activity in rat platelet membrane fractions.



Samples of platelet membrane preparations (0.3 mg in 1.0 ml) were incubated at 37°C for 20 min containing FFA albumin (0.5 mg/ml) at pH 9.0.

Figure 4.4. Dependence of phospholipase A_2 activity in rat platelet membrane fractions on membrane protein concentration



Membrane samples (1.0 ml) were incubated for 20 min at 37°C [Ca^{2+}] = 2.0 mM, FFA albumin (0.5 mg/ml) pH = 9.0.

concentration to use in this assay because the rate of conversion of substrate to product was sufficiently high, and this was approximately the physiological concentration that may be found within the cell (Pickett et al., 1977). Figure 4.4 shows that phospholipase A_2 activity was not linearly related to the concentration of membrane protein present, therefore in subsequent assays a standard amount of protein was used. This non linearity is not unusual in membrane bound proteins and this was found with acyl coenzyme A transferase (Rae, 1980).

4.7 Discussion

Experiments have been carried out in the present study to determine suitable conditions for a phospholipase A_2 assay technique in both platelet membrane preparations and whole platelets. It was necessary to investigate the effect of Ca^{2+} concentration on the phospholipase A_2 activity of membrane preparations. This was because several workers who have studied this enzyme used a wide range of Ca^{2+} concentrations. Kannagi and Koizumi (1979) studied the activity of a purified form of phospholipase A_2 from rabbit platelets with 0.01 mM Ca^{2+}. Rittenhouse-Simmons and Deykin (1978) used 10 mM Ca^{2+} with platelet lysates, and Trugnan et al. (1979) used 10 mM Ca^{2+} with human platelet membrane fractions. Wong and Cheung (1979) used 0.2 mM Ca^{2+} in their studies of phospholipase A_2 activity in membrane preparations. In the present study, 0.2 mM Ca^{2+} was found to be too low for membranes prepared from rat platelets.

The results shown in figure 4.1 suggested that fatty acid free albumin was required in the incubation medium for optimum conversion of [1-^{14}C]oleoyl PC to [1-^{14}C]oleic acid. Stuart et al (1980b) showed that when human platelets prelabelled with [1-^{14}C] arachidonic acid were stimulated with thrombin (5.0 U/ml), 28 per cent of this arachidonic acid was released in the presence of albumin (0.7 g/100 ml) compared to 18 per cent without albumin.

Kupferberg et al (1981) showed that the activity of phospholipase A_2 (from Crotalus atrox) was inhibited by lysolecithin which is a product of the hydrolysis of PC catalysed by this enzyme as a result of the formation of a lysolecithin-phospholipase A_2 complex. Kupferberg et al. demonstrated that bovine serum albumin bound to the lysolecithin mitigating its inhibitory activity providing the albumin was present in excess. The single assays carried out using two different concentrations of albumin (Figure 4.1) tend to confirm that albumin serves to stimulate phospholipase A_2 activity in rat platelet membrane fractions. This is presumed to occur possibly by way of abolishing the inhibitory effect of products as seen in studies of phospholipase A_2 isolated from Crotalus atrox by Kupferberg et al. (1981). Alternatively the albumin could act by combining with the substrate and delivering it to the enzyme more efficiently.

The buffer in which crude membrane preparations were finally dispersed was pH 9. Many investigators have reported that the pH optimum for phospholipase A_2 activity was in the range 8.0 to 10.5 (see Jesse and Franson, 1979). As mentioned above, the concentration of Ca^{2+} for resuspended platelets was 0.2 mM because above this concentration spontaneous aggregation frequently occurred on stirring. It was necessary to use platelets resuspended in a suitable buffer so that lipid extraction (for the phospholipase A_2 assay) was possible. It was necessary to use these unphysiological conditions because extraction of lipids from platelets in plasma would have been extremely difficult due to the very high protein concentration in the sample. The method of prelabelling platelets with radiolabelled fatty acids was not found to be suitable. The assay technique developed for resuspended platelets and platelet membrane preparations has been used in further investigations of the effect of the platelet membrane of

alteration of cholesterol content and cholesterol-analogue incorporation. In view of the finding by several workers that the activities of other membrane bound enzymes are influenced by their lipid environments (McMurchie and Raison, 1979; Agutter and Suckling, 1981), these further investigations of phospholipase A_2 were very pertinent to the present project.

Chapter 5

Aggregation and Phospholipase A_2 Studies in once Resuspended Rat Platelets

5.1 Introduction

Phospholipase A_2 is considered to play an important role in the platelet membrane in the regulation of release of arachidonic acid from platelet membrane phospholipids (Blackwell et al., 1977). After hydrolysis of membrane phospholipids, arachidonic acid is made available for metabolism to PGH_2 and thromboxane A_2 which are believed to be important potent proaggregating agents (see Chapter 1). In a study related to the present work, Stuart et al. (1980a) altered the cholesterol content of human platelets *in vitro* by the method of Shattil et al. (1975).

Stuart et al. (1980a) incubated human platelets with liposomes to either increase or decrease the platelet cholesterol content. To the same incubation system, [1-^{14}C] arachidonic acid was added in order to label the platelet phospholipids. After incubation for 5 hours the platelets were washed to remove liposomes and [^{14}C] arachidonic acid not incorporated into platelet phospholipids. Platelet samples were then treated with thrombin. Control samples were treated with buffer alone. The results obtained by Stuart et al. (1980a) showed that in cholesterol-enriched platelets there was increased synthesis of cyclo-oxygenase products and slightly reduced synthesis of lipoxygenase products compared to cholesterol-depleted platelets after thrombin induced aggregation. These workers suggested that there was an increased release of [^{14}C] arachidonic acid from prelabelled phospho-lipids in the cholesterol-enriched platelets. The amount of arachidonic acid released was calculated by determining the percentage of the radioactivity in the arachidonic acid and metabolite fractions and subtraction of the values obtained when buffer instead of thrombin was added to the platelets. Stuart et al. (1980a) did not however state if there was any difference in the amount of [^{14}C] arachidonic

acid taken up by cholesterol-depleted or cholesterol-enriched platelets during the 5 hour incubation period. Also, no mention was made of the percentage release of [^{14}C] arachidonic acid from platelets incubated with buffer instead of thrombin. If there was any difference in either of these parameters between the cholesterol-depleted and cholesterol-enriched platelets, the suggestion that there was a greater release of [^{14}C] arachidonic acid from cholesterol-enriched platelets on stimulation with thrombin is not well supported.

The suggestion by Stuart et al. that there was "a serious derangement of arachidonic acid metabolism" is however well founded. The differences in percentages of total radioactivity found in metabolites of the lipoxygenase and cycloxygenase pathways indicated that arachidonic acid metabolism was directed preferentially toward the cyclo-oxygenase pathway in the cholesterol-enriched platelets. These workers suggested that membrane cholesterol may affect the enzymes diacylglycerol lipase and/or phospholipase A_2. Both these enzymes are involved in the release of arachidonic acid from platelet phospholipids (Billah et al., 1980). The methods used by Stuart et al. (1980a) to determine the per cent release of arachidonic acid would not have distinguished between these two enzyme systems. It was therefore necessary to investigate further the effect of cholesterol enrichment on platelets using a technique designed to specifically assay for phospholipase A_2.

Recent research has revealed that there are at least two mechanisms for the release of arachidonic acid from platelet membrane phospholipids (Billah et al., 1980). One mechanism involves the hydrolysis of the sn-2-acyl ester bond of phospholipids by phospholipase A_2. The other involves a phospholipase C which cleaves phosphatidylinositol at the phosphate bond to produce the corresponding 1,2 diacylglycerol and inositol phosphate (Rittenhouse-Simmons, 1979). The 1,2 diacylglycerol is then

either phosphorylated by diacylglycerol kinase to form phosphatidic acid (Call and Rubert, 1973; Billah et al., 1979) or degraded further by a diacylglycerol lipase to release the monoacylglycerol and arachidonic acid (Bell et al., 1979). It has been shown by Lapetina and Cuatrecasus (1979) that platelets stimulated with thrombin rapidly form phosphatidic acid from phosphatidylinositol within 5 seconds prior to the appearance of arachidonic acid. Phosphatidic acid production may occur in stimulated platelets before phospholipase A_2 activation, and may in some way activate this enzyme (Lapetina and Cuatrecasus, 1979; Lapetina et al., 1980).

It has been suggested by Bell et al. (1979) that sufficient arachidonate may be provided by the combined activities of phospholipase C and diacylglycerol lipase for the synthesis of prostaglandins and thromboxanes on stimulation of platelets with thrombin. The phospholipase C is reported to be specific for phosphatidyl inositol (Rittenhouse-Simmons, 1979; Billah et al., 1979; Bell et al., 1979) and is also called phosphatidylinositol-phosphodiesterase. It had been reported previously by other workers that phospholipase A_2 activity was responsible for the release of arachidonic acid (Bills et al., 1976; Blackwell et al., 1977; Lapetina et al., 1977; Bills et al., 1978; Lapetina et al., 1978). The view generally accepted to date is that phospholipase C activation is an early event following platelet stimulation, and phospholipase A_2 is activated subsequently (Lapetina and Cuatrecasus, 1979).

Platelet phospholipase A_2 activity has been investigated by several workers (see for example Pickett et al., 1977 and Rittenhouse-Simmons and Deykin, 1978), and it is quite clear that phospholipase A_2 plays an important role in arachidonic acid metabolism in platelets, and Broekman et al. (1980) suggested that the "phosphatidylinositol-specific"

and phospholipase A_2 pathways of phospholipid metabolism exist in stimulated platelets. It seems unlikely therefore that arachidonic acid is released exclusively by phosphatidylinositol phospholipase C as suggested by Bell et al. (1979). It is generally considered that phospholipase C specifically cleaves the phosphate ester bond of phosphatidylinositol whereas the major substrates for phospholipase A_2 are reported to be phosphatidylethanolamine (Broekman et al., 1980; Jesse and Cohen, 1976) and phosphatidylcholine (see for example Bills et al., 1977). Phospholipase C is a cytosolic protein whereas phospholipase A_2 is a membrane bound enzyme (Billah et al., 1980). It was of particular interest in the present study to investigate the activity of phospholipase A_2 in platelets with altered cholesterol content in order to examine the possibility of increased release of arachidonic acid as a result of alteration of the physical properties of the membrane. Since there are two pathways by which arachidonic acid may be released from platelet membrane phospholipids, as discussed above, an important criterion in an assay of phospholipase A_2 activity is that the substrate presented to platelets should not be available to phospholipase C activity. The method for the assay of phospholipase A_2 described in Chapter 4 satisfies this condition. [1-^{14}C] Oleoyl PC would not be a substrate for phospholipase C because of its specificity for phosphatidylinositol. Using the method described in Chapter 4 for the assay of phospholipase A_2 activity the oleic acid released would not be available for further metabolism by enzymes normally involved in arachidonic acid metabolism, and would therefore give a good index of general phospholipase A_2 activity.

In Chapter 4, results were presented which indicated that it was feasible to assay phospholipase A_2 activity in once resuspended rat

platelets. In this chapter experiments which have been carried out to investigate the relation between phospholipase A_2 activity and sensitivity to aggregation are reported. Firstly however the difficulties involved in measuring platelet aggregation will be described.

5.2. Platelet Aggregation studies

The general background (see Chapter 1) and the methodology used to test aggregation (see Chapter 2) have already been described. Preliminary initial aggregation studies were carried out using ADP with human platelets (see Figure 7.1, Chapter 7) but subsequently the use of collagen was adopted. Although either agent could be used to test aggregation, collagen was considered the more suitable. Collagen is a strong agonist of aggregation which is capable of stimulating the basic platelet reaction to the final event of granule secretion. ADP is a comparatively weak agonist which although stimulates aggregation does not induce granule secretion as discussed in Chapter 1.

Initial observations showed that the rate and per cent maximum extent of aggregation measured essentially the same thing (see for example Table 5.4A and 5.4B). The extent of aggregation has subsequently been measured in place of rate of aggregation. This precluded the theoretical possibility that two platelet samples could show the same rate when in one sample the platelets aggregated sooner but to a lesser extent than in the other sample. During the current studies however this phenomenon has not been observed.

In the present studies, it is evident that platelets isolated from different groups of rats showed variable sensitivity to collagen and ADP induced aggregation after three hours incubation with liposomes. This is summarized in Figure 5.1 which shows all the results of extent of aggregation induced by the various concentrations of collagen used in samples of rat platelets incubated with cholesterol-normal liposomes. The variation seen from one experiment to another may be due to a

Figure 5.1. Variation in sensitivity of platelet samples from different groups of rats to collagen induced aggregation.

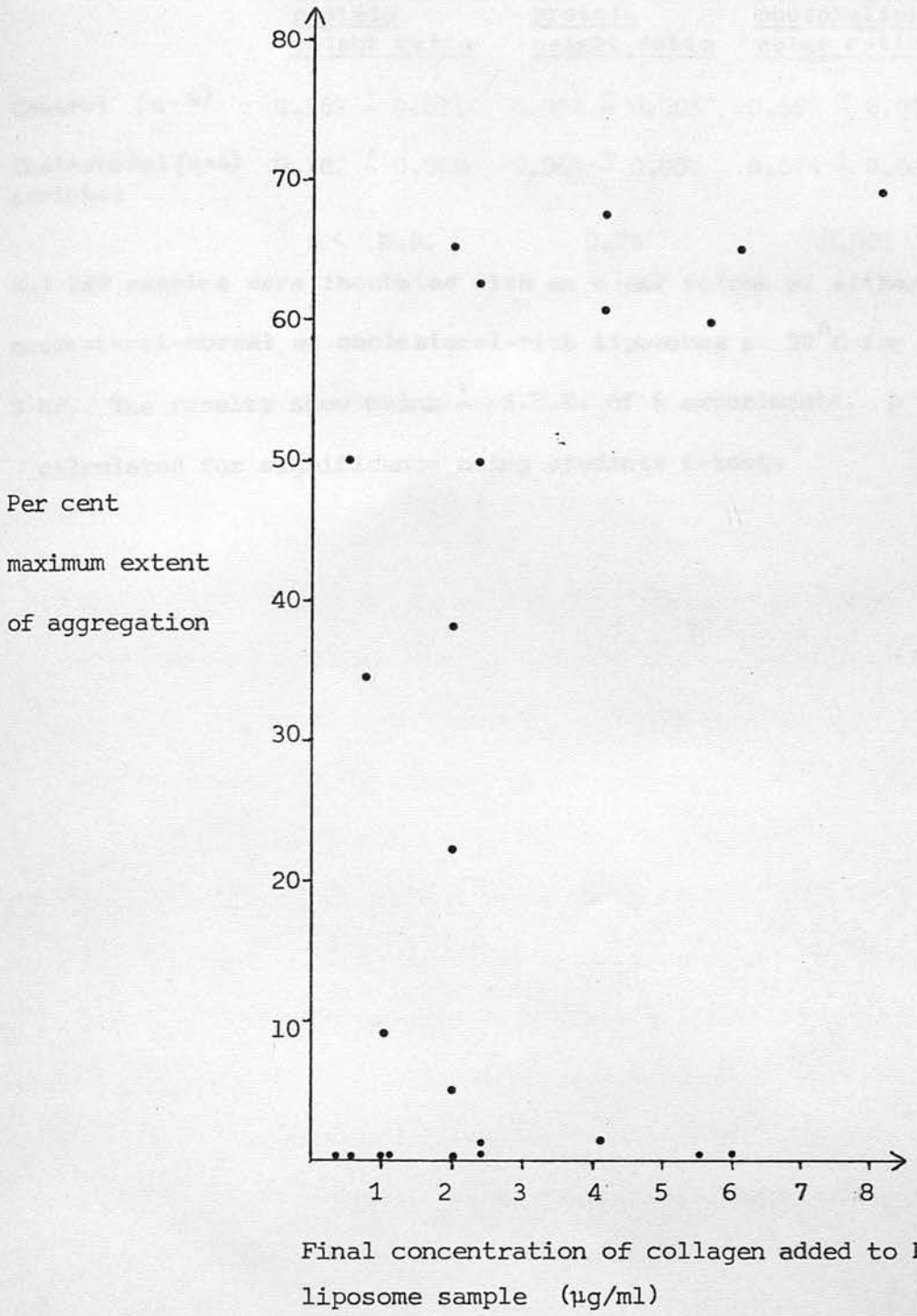


Figure 5.1 shows extent of aggregation of platelets incubated with cholesterol-normal liposomes after 3 hrs incubation. This figure summarizes data collected from all the experiments reported in this thesis. Aggregation tests were carried out as described in Chapter 2.

Table 5.1. Analysis of rat platelets

	Phospholipid: protein weight ratio	Cholesterol: protein weight ratio	Cholesterol: phospholipid molar ratio
Control ($n=6$)	0.187 ± 0.011	0.054 ± 0.005	0.567 ± 0.035
Cholesterol enriched ($n=6$)	0.182 ± 0.008	0.062 ± 0.005	0.674 ± 0.028
$p <$	N.S.	0.05	0.001

Rat PRP samples were incubated with an equal volume of either cholesterol-normal or cholesterol-rich liposomes at 37°C for 3 hr. The results show means ± S.E.M. of 6 experiments. p values were calculated for significance using students t-test.

difference in sensitivity of platelets from different groups of rats, and this sensitivity may be further altered by the incubation process. Therefore before commencing aggregation studies for each experiment, it was necessary to test platelet samples with a wide range of collagen concentrations in order to find one intermediate between no response and maximum stimulation.

Six experiments were carried out to assay phospholipase A_2 activity in resuspended control and cholesterol-enriched rat platelets. Platelets were enriched with cholesterol or alternatively in control platelets the normal cholesterol content was maintained using the incubation method with liposomes as described in Chapter 3. The six experiments were divided into two groups of three. In the first group (Tables 5.1A-5.3B) platelets were resuspended once at the end of the incubation period and were assayed for phospholipase A_2 activity by incubation in a shaking waterbath at 37°C without added collagen. In the second group (Tables 5.4A-5.6B) collagen was added to resuspended platelet samples in order that aggregation could be recorded in the sample being assayed for phospholipase A_2 activity. The results in Table 5.1 give the means of analysis of platelet samples from the six experiments carried out. The mean increase in the cholesterol:phospholipid molar ratio was 20 per cent in the cholesterol-enriched platelets. Aggregation studies were carried out on PRP-liposome samples in all the experiments, and samples of PRP-liposome mixtures were taken for counting to ensure the platelet counts were not significantly different.

1. Experiment 1. (Tables 5.1A and 5.1B)

In Table 5.1A, the results show hypersensitivity to collagen induced aggregation in cholesterol-enriched platelets. This was particularly obvious with a collagen concentration of 2.0 µg/ml where control

EXPERIMENT 1

Table 5.1A. Aggregation results of PRP-liposome samples (0.5 ml)

	Aggregation % max. extent			Platelet count in sample tested x 10^9/litre
Collagen final concentration µg/ml	1.6	2.0	2.4	
Control	0	0	50	594
Cholesterol-rich	4	41	66	590

Aggregation tests were carried out as described in Chapter 2.

Table 5.1B. Phospholipase A_2 assay of resuspended rat platelets

Control Platelets		Cholesterol-rich platelets	
Platelet count x 10^9/litre	Phospholipase A_2 activity % conversion	Platelet count x 10^9/litre	Phospholipase A_2 activity % conversion
1420	14.8	1130	13.0
710	24.5	560	15.5
350	51.5	280	14.0
170	42.0	140	33.0
85	37.0	70	24.0

Samples of platelets of different count were obtained by resuspending platelets in half the original volume (in PRP) and diluting with buffer. Phospholipase A_2 assay was carried out by the addition of [1-^{14}C] oleoyl PC (10 nCi). [Ca^{2+}] = 0.2 mM; FFA albumin concentration = 3.5 mg/ml; incubation time = 40 min at 37°C; Glucose = 5 mM; pH = 7.4.

Results shown in Tables 5.1A and 5.1B are for single tests only

EXPERIMENT 2

Table 5.2A. Aggregation results of PRP-liposome samples

	Aggregation % max. extent		Platelet count x 10^9/litre
Collagen final concentration µg/ml	1.0	2.0	
Control	0	22	634
Cholesterol-rich	10	48	641

0.25 ml PRP-liposome samples were tested for aggregation as described in Chapter 2.

Results shown are for single tests only

Table 5.2B. Phospholipase A_2 assay of samples of resuspended control and cholesterol-rich rat platelets of different platelet counts

Platelet count x 10^9/litre	Phospholipase A_2 activity % conversion	
	Control platelets	Cholesterol-rich platelets
450	37.7	35.4
225	34.0	30.5
113	17.8	20.0

Phospholipase A_2 assays were carried out using [1-^{14}C] oleoyl PC (10 nCi) as substrate. The results given are for single tests only $[Ca^{2+}]$ = 0.2 mM; incubation time = 15 min at 37°C; FFA albumin concentration = 3.5 mg/ml; [Glucose] = 5 mM; pH 7.4.

platelets failed to aggregate and cholesterol-enriched platelets aggregated to 41 per cent of the maximum extent. Phospholipase A_2 assays were carried out on samples of resuspended platelets of different platelet count. These samples were obtained by serial dilution with modified Tyrode's buffer. The results may suggest a higher phospholipase A_2 activity in the control platelets, but a direct comparison was not possible because the counts were not the same in both samples.

2. Experiment 2. (Tables 5.2A and 5.2B)

The results in Table 5.2A show that cholesterol-enriched platelets were more sensitive to collagen induced aggregation than control platelets. Phospholipase A_2 activity (see Table 5.2B) was assayed in platelet samples of different platelet counts. This was a repeat of experiment 1, except that in this experiment, control and cholesterol-enriched platelet samples contained the same platelet count. The results indicate no obvious difference in phospholipase A_2 activity between the control and cholesterol-enriched platelets. A shorter incubation period was used in order to reduce the amount of substrate converted to product, so that substrate removal would not limit the rate of reaction. However, the per cent conversion remained high (above 20 per cent in most samples) which was not desirable.

3. Experiment 3. (Tables 5.3A and 5.3B)

The results in Table 5.3A indicate that cholesterol-enriched platelets were more sensitive to collagen induced aggregation than control platelets. In this experiment phospholipase A_2 activity was assayed at intervals during an incubation at $37^{\circ}C$ in both control and cholesterol-enriched platelets. The results suggest the possibility that phospholipase A_2 activity was detectable at an earlier stage in cholesterol-enriched platelets than in control platelets.

EXPERIMENT 3

Table 5.3A. Aggregation results of PRP-liposome samples (0.25 ml)

	Aggregation % max. extent	Platelet count x 10^9/litre
Collagen final concentration µg/ml	2.4	
Control	0	641
Cholesterol-rich	52	676

Table 5.3B. Time course for phospholipase A_2 activity in resuspended control and cholesterol-enriched rat platelets

	Phospholipase A_2 activity % conversion	
Time (min)	Control	Cholesterol-rich
1	0.35	3.5
15	0.0	3.8
30	3.4	4.3
50	5.4	3.3
70	6.4	6.3
Platelet count x 10^9/litre	637	575

Phospholipase A_2 assays were carried out using [1-^{14}C] oleoyl PC (10 nCi) as substrate. [Ca^{2+}] = 0.2 mM; incubation at 37°C; FFA albumin concentration = 3.5 mg/ml; [Glucose] = 5 mM; pH = 7.4.

Results shown in Tables 5.3A and 5.3B are for single tests only

However, the per cent conversions in all the samples was low, and this could have hidden any small differences that may have existed in the phospholipase A_2 activity.

The results obtained from the three experiments discussed above do not indicate an obvious difference in phospholipase A_2 activity in unstimulated platelets. It was shown in Chapter 4 (Table 4.3) that resuspended platelets could be stimulated with collagen and the phospholipase A_2 activity could be assayed in the same sample. The results indicated a possible correlation between concentration of collagen and phospholipase A_2 activity. In the three experiments described above, cholesterol-enriched platelets in PRP-liposome mixtures were consistently more sensitive to collagen induced aggregation. The next three experiments therefore centred on assaying phospholipase A_2 activity in once resuspended platelets which were stimulated to aggregate with collagen.

4. Experiment 4. (Tables 5.4A and 5.4B)

The results in Table 5.4A show the enhanced sensitivity of cholesterol-enriched rat platelets to collagen induced aggregation. The rate of aggregation is given in brackets, and this was also enhanced. The results in Table 5.4B show that in once resuspended platelets, cholesterol enriched platelets are less sensitive to collagen induced aggregation than control platelets. The rate of aggregation is also reduced. Phospholipase A_2 activity was not different between the two samples.

5. Experiment 5. (Tables 5.5A and 5.5B)

The results obtained in experiment 5 confirmed the results from experiment 4. Cholesterol enriched platelets in PRP-liposome samples were more sensitive to collagen induced aggregation than control platelets (Table 5.5A), but were less sensitive after resuspension

EXPERIMENT 4

Table 5.4A. Aggregation results of PRP-liposome samples

	Aggregation % max. extent (Rate of aggregation min^{-1})		Platelet count x 10^9/litre
Collagen final concentration µg/ml	1.0	2.0	
Control	9.2(3.4)	Irreversible	479
Cholesterol-rich	19.0(8.5)	Irreversible	472

Results shown are for single tests only

Aggregation results in brackets are rates of aggregation.

See Chapter 2 for details of method.

Table 5.4B. Aggregation results and phospholipase A_2 assays of resuspended control and cholesterol-rich rat platelets

	Aggregation % max. extent	Phospholipase A_2 activity % conversion	Platelet count x 10^9/litre
Collagen final concentration µg/ml	2.0		
Control	82(21)	5.3 ± 0.87	380
Cholesterol-rich	53(13)	4.7 ± 0.32 $p <$ N.S.	395

Aggregation results are for single tests only. Platelets were resuspended in an equivalent volume of buffer and phospholipase A_2 assays were carried out in triplicate using [1-^{14}C] oleoyl PC (10 nCi) as substrate. [Ca^{2+}] = 0.2 mM; incubation time = 10 min at 37°C; FFA albumin concentration = 3.5 mg/ml; [Glucose] = 5 mM; pH = 7.4.

EXPERIMENT 5

Table 5.5A. Aggregation results of PRP-liposome samples

	Aggregation % max. extent to collagen (1.2 µg/ml)	Platelet count x 10^9/litre
Control	0	450
Cholesterol-rich	60	430

Samples (0.25 ml) were tested for aggregation as described in Chapter 2. Results shown are for single tests only

Table 5.5B. Aggregation results and phospholipase A_2 assays of resuspended control and cholesterol-rich rat platelets

	Aggregation % max. extent	Phospholipase A_2 activity % conversion	Platelet count x 10^9/litre
Collagen final concentration µg/ml	2.0		
Control	62 ± 3.16	3.5 ± 0.29	289
Cholesterol-rich	28 ± 1.73	3.5 ± 0.79	246
	$p < 0.001$	N.S.	

Aggregation tests and phospholipase A_2 assays were carried out in triplicate samples from each incubation system. Phospholipase A_2 assays were carried out using [1-^{14}C]arachidonoyl PC (10 nCi) as substrate. Results show means ± S.E.M. Significance was tested using students t-test. Phospholipase A_2 assay conditions:- [Ca^{2+}] = 0.2 mM; incubation time = 10 min at 37°C; FFA albumin concentration = 3.5 mg/ml; [Glucose] = 5 mM; pH = 7.4.

EXPERIMENT 6

Table 5.6A. Aggregation results of PRP-liposome samples

	Aggregation % max. extent		Platelet count x 10^9/litre
Collagen final concentration µg/ml	2.4	4.0	
Control	0	67	498
Cholesterol-rich	40.5	65	499

Samples (0.25 ml) were tested for aggregation as described in Chapter 2.

Results shown are for single tests only

Table 5.6B. Phospholipase A_2 assay of resuspended control and cholesterol-rich rat platelets

	Phospholipase A_2 activity % conversion	Platelet count x 10^9/litre
Control	5.8 ± 0.50	499
Cholesterol-rich	6.1 ± 0.71	451
	p < N.S.	

Phospholipase A_2 assays were carried out using [1-^{14}C] oleoyl PC (10 nCi) as substrate. Collagen was added to each sample in a final concentration of 2 µg/ml. Results show means ± S.E.M. for single assays carried out on 6 samples isolated from one control PRP-liposome incubation, and 6 from one cholesterol enriching PRP-liposome incubation. [Ca^{2+}] = 0.4 mM; incubation time = 10 min at 37°C; FFA albumin concentration = 3.5 mg/ml; [Glucose] = 5 mM; pH 7.4.

compared to controls. [1-^{14}C] arachidonoyl PC was used as substrate for the phospholipase A_2 assay to test the possibility that [1-^{14}C] oleoyl PC was not a suitable substrate which could have prevented the detection of any difference in activity of this enzyme. No difference was detected even with this substrate, suggesting that the substrate [1-^{14}C] oleoyl PC was equally suitable as [1-^{14}C] arachidonyl PC in this system of phospholipase A_2 assay.

6. Experiment 6. (Tables 5.6A and 5.6B)

The results in Table 5.6A again confirmed that cholesterol-enriched rat platelets in PRP-liposome samples were more sensitive to collagen induced aggregation than control platelets. Six samples each of the resuspended control platelet sample and of the resuspended cholesterol enriched platelet sample were assayed for phospholipase A_2 activity. The Ca^{2+} concentration was raised to 0.4 mM to test whether any difference in phospholipase A_2 activity was detectable under these conditions. Collagen was added to each sample at a final concentration of 2 μg/ml which was the concentration used in the previous two experiments. All samples aggregated irreversibly probably due to the high Ca^{2+} concentration used. The range of values obtained for per cent conversion in the two groups overlapped considerably, indicating no difference in phospholipase A_2 activity under these conditions.

5.3 Discussion

In all six experiments, cholesterol-enriched platelets in PRP-liposome samples were consistently more sensitive to collagen induced aggregation than control platelets treated in the same way. The results for phospholipase A_2 activity suggest that there was no detectable difference in the activity of this enzyme in cholesterol-enriched platelets compared to control platelets, either when stimulated with collagen or unstimulated. The results obtained from the last three experiments (Tables 5.4A-6B) regarding aggregation testing of

resuspended platelets show that the sensitivity of cholesterol-enriched platelets to collagen induced aggregation was reduced. This sensitivity was less than for control platelets treated in the same way. In samples of resuspended platelets tested for aggregation, the aggregation pattern was distinctly different from the normal pattern. The initial swelling phase, which is easily recognised in normal collagen induced aggregation traces, was lost. When aggregation occurred it was always irreversible. These results are in agreement with Manucci (1972), who showed that the aggregation pattern of resuspended platelets was altered in this way.

It was possible that the resuspending procedure activated cholesterol-enriched platelets to a greater extent than control platelets. If this was the case, the cholesterol-enriched platelets would then become less responsive to stimuli, giving rise to the observed decreased aggregation extent. It remains a possibility therefore that the activity of phospholipase A_2 would be diminished correlating with the decreased sensitivity to collagen. The level of phospholipase A_2 activity detected by this method may therefore have been artificially low in the cholesterd-enriched platelets. No difference in phospholipase A_2 activity was detected in platelets which were not stimulated, and as previously mentioned, it is possible that any difference in activity of this enzyme would occur when platelets were stimulated by agents inducing thromboxane synthesis.

In view of these findings, it was considered that the method of phospholipase A_2 assay in once resuspended platelets was unsuitable. Therefore an alternative method for phospholipase A_2 assay was developed to avoid the problems encountered with resuspended platelets. The method developed involved using a crude platelet membrane fraction as a source of phospholipase A_2 as described in Chapter 4. This method was subsequently used to investigate phospholipase A_2 activity in human

platelet membranes (Chapter 7) and rat platelet membranes of various sterol compositions (Chapter 8). The technique of assaying for phospholipase A_2 activity in once resuspended platelets was reinvestigated in freshly obtained platelets from rabbits (Chapter 6), in order to avoid the 3 hour incubation period used in the experiments described in this chapter which could be responsible in part for loss of platelet sensitivity.

Chapter 6

Investigations of Platelets from Rabbits Fed a Cholesterol Rich Diet

6.1 Introduction

It is generally accepted that subjects with a high serum cholesterol level may be at greater risk of developing atherosclerosis than subjects with a normal serum cholesterol level (Marx, 1976). Altered platelet function has been implicated as a contributing factor in the development of thrombosis and atherosclerosis (see for example Colman, 1978; Lees and Carvalho, 1978). Carvalho et al. (1974a) investigated the function of platelets from type IIa hyperbetalipoproteinaemic subjects (type IIa). Subjects diagnosed with this disease have abnormally high serum levels of cholesterol and LDL (Lees et al., 1973) as discussed in Chapter 1. It was shown by Carvalho et al. (1974a) that platelets from type IIa subjects were more sensitive to aggregation induced in vitro with adrenaline, ADP and collagen than normal platelets. In a preliminary report by Bennett et al. (1974) and in a later report by Shattil et al. (1977) it was shown that platelets from type IIa subjects contained a cholesterol:phospholipid molar ratio 7 per cent higher than platelets from normal subjects. Platelets from type IIa subjects were shown to be hypersensitive to aggregation induced by ADP and adrenaline.

Many researchers have investigated the pathogenesis of atherosclerosis using the approach of studying the disease induced in animal models. Some workers have induced atherosclerosis by mechanically damaging endothelial tissue in rats (Hornstra et al., 1973) and in rabbits (Weigensburg et al., 1975; Day et al., 1974). Other workers have attempted to induce atherosclerosis or thrombosis in animals by feeding them atherogenic diets. Thomas and Hartcroft (1959) fed rats a diet containing an abnormally high proportion of fats including cholesterol. Thiouracil and sodium cholate were included in this diet so that blood cholesterol levels would rise. The diet induced a significant increase

in the serum cholesterol level in these rats giving rise to a higher incidence of myocardial infarctions than in rats fed a normal diet. Frost (1969) fed rabbits a diet rich in cholesterol for 7 weeks. After this time, numerous platelets were observed to have adhered to the wall of the abdominal aorta in tissue sections prepared from the carcasses of these rabbits. Aortic sections prepared from rabbits fed a normal diet showed no signs of platelet adherence.

The results of the studies just discussed show that dietary lipid composition influences the cholesterol level in the blood, and appears to affect platelet function. The finding that platelet activity was increased in a cholesterol-rich environment in animal models was consistent with the findings in hypercholesterolaemic human subjects. The studies in animal models discussed above did not however investigate platelet function in detail.

Joist et al. (1976) induced hyperlipidaemia in rabbits by feeding them a diet enriched with egg yolk for 16 weeks. These workers showed that platelets from the hyperlipidaemic rabbits resuspended in Tyrode's albumin solution showed a significant increase in collagen and thrombin induced aggregation compared with similarly treated platelets from rabbits fed a control diet. The plasma cholesterol level was 617 mg/100 ml compared to 17 mg/100 ml in control rabbits. In this investigation by Joist et al., analysis of platelet cholesterol content was not carried out. It is not possible to determine therefore, whether the high serum cholesterol level influenced the platelet cholesterol content *in vivo*, and whether this might have been a factor influencing platelet function. In the light of evidence from Shattil et al. (1977) that platelets from human type IIa subjects had a raised cholesterol:phospholipid molar ratio, it was of interest in the present study to analyse platelets from animals with induced hypercholesterolaemia

to observe whether the platelet cholesterol content is influenced by the cholesterol level in the plasma environment.

A clear indication of the effect on platelet cholesterol content of feeding animals a cholesterol-rich diet was reported by Yoshida et al. (1977). These workers fed a cholesterol-rich diet to guinea pigs for 8 weeks and showed that platelets from animals fed this diet became increasingly sensitive to ADP induced aggregation during the dietary regimen compared to platelets from animalsmaintained on a normal diet. Yoshida et al. (1977) analysed platelets for cholesterol and phospholipid content. Calculation based on the results they presented show that cholesterol:phospholipid molar ratio of platelets from guinea-pigs fed the cholesterol diet was increased by approximately 40 per cent after 8 weeks compared to platelets from the control group. The results presented by this group demonstrated that cholesterol feeding not only increased the serum cholesterol level, but also raised the platelet cholesterol content. The enhanced activity seen in the platelets from the cholesterol fed animals may in part be due to the cholesterol enrichment.

Some researchers have attempted to explain why platelets become hyperactive in atherosclerosis. Srivastava (1974) studied the synthesis of prostaglandins (PGE_1, PGE_2, $PGF_{2\alpha}$) by platelets isolated from rabbits fed a normal diet. Since PGE_1 is a potent anti-aggregating agent, and PGE_2 is a mild pro-aggregating agent, Srivastava considered the possibility that the capacity of the platelet to synthesize these prostaglandins could be altered. In fact, no differences were detected. The results showed great variations in amounts of prostaglandins synthesized by platelets from individual rabbits within the same group, and this may have tended to hide any small differences that could have existed between the two groups. More importantly,

the prostaglandins studied by Srivastava may play much less significant roles in platelet aggregation than thromboxane A_2 which was identified more recently (Hamberg et al., 1974). It was shown by these workers that two orders of magnitude more thromboxane B_2 (a degradation product of thromboxane A_2), HHT and HETE were produced by human platelets than $PGF_{2\alpha}$ or PGE_2.

More recently, research into atherosclerosis by the approach of studying animals fed a cholesterol-rich diet has focussed more closely on investigations into arachidonic acid metabolism. It has been shown that platelets synthesised the potent pro-aggregating agent thromboxane A_2 from arachidonic acid (Hamberg et al., 1975). More recently, it was recognised that arachidonic acid was metabolised by arterial and venous tissues into a potent anti-aggregating agent, prostacyclin (Moncada et al., 1976; Gryglewski et al., 1976; Moncada et al., 1977). Researchers became more interested in arachidonic acid metabolism in atherosclerosis because it was suggested that there may exist an intricate balance between the synthesis of these two compounds (Bailey, 1979). On the one hand, thromboxane A_2 synthesised by platelets promotes aggregation and vasoconstriction, whereas prostacyclin synthesised by endothelial tissue prevents platelet clumping and reduces vasomotor tone thus inducing vasodilatation.

Dembinska-Kiec et al. (1977) investigated the generation of prostacyclin by coronary vascular bed tissues isolated from atherosclerotic rabbits compared to these tissues isolated from normal rabbits. Atherosclerosis was induced by feeding a group of rabbits a normal diet supplemented with 1 per cent cholesterol, and prostacyclin production was significantly reduced in coronary vascular tissue from these animals compared to control animal coronary vascular tissue. These findings suggested that atherosclerosis induced by cholesterol feeding upset the intricate balance between production of thromboxane A_2 and

prostacyclin. Subsequently Zmuda et al. (1977) showed that platelets from atherosclerotic rabbits (induced by feeding a cholesterol-rich diet) were more sensitive to arachidonic acid induced aggregation, and that thromboxane A_2 generation was increased compared to platelets from animals fed a normal diet. A more critical examination of arachidonic acid metabolism in cholesterol fed atherosclerotic rabbits was carried out by Gryglewski et al. (1978). Rabbits were fed a 1 per cent cholesterol diet for 5 months, and arachidonic acid induced aggregation of platelets in PRP was found to be increased at 3 months compared to platelets from control animals. Prostacyclin production induced with arachidonic acid in perfused hearts and spontaneous generation of prostacyclin by mesenteric arteries and aortae were strongly suppressed after 1 and 3 months from rabbits fed the cholesterol-enriched diet compared to normal control rabbits. Platelet sensitivity to the pro-aggregating action of ADP and to the anti-aggregating action of prostacyclin was increased after 1 month on the cholesterol-enriched diet. Gryglewski suggested therefore that the first stage of atherosclerosis may be casually related to the strong suppression of prostacyclin generation by arteries, whereas arachidonic acid metabolism in platelets remains unchanged. Gryglewski also suggested that in the early stage of atherosclerosis the increased susceptibility of platelets to ADP and prostacyclin may be due to the lowering of cAMP levels in platelets as a consequence of prostacyclin deficiency. Dembinska-Kiec et al. (1979) have investigated further platelets from atherosclerotic rabbits. These workers showed that intact platelets from atherosclerotic rabbits (induced by cholesterol diet) synthesised more thromboxane B_2 and HETE than platelets from normal rabbits when incubated with [^{14}C] arachidonic acid. No difference was found in the synthesis of these metabolites by platelet homogenates from normal and atherosclerotic animals.

These results suggested that the pathway synthesising thromboxane A_2 from arachidonic acid is more active in whole platelets from atherosclerotic rabbits compared to whole platelets from normal rabbits. This may reflect enhanced activities of the cyclo-oxygenase and thromboxane synthetase enzymes. The increased HETE production also observed indicated an enhanced lipoxygenase activity.

In the four studies discussed above, there are several important points with regard to the present study. In all the experiments discussed above (Dembinska-Kiec et al., 1977; Zmuda et al., 1977; Gryglewski et al., 1978; Dembinska-Kiec et al., 1979) this research team have fed rabbits a diet supplemented with 1 per cent cholesterol, and atherosclerosis was confirmed by post mortem examination. However, neither blood cholesterol levels, nor the cholesterol content of platelets were monitored during these experiments. In all the studies in platelets of metabolism of arachidonic acid, exogenous arachidonic acid was added to platelet preparations. The release of arachidonic acid from platelet phospholipids is considered to be a critical step in the generation of thromboxane A_2 (Blackwell et al., 1977). In the studies discussed above this reaction has not been tested, so it is not possible to say whether the platelets from atherosclerotic rabbits were capable of synthesising greater quantities of thromboxane A_2 derived from membrane phospholipids containing arachidonic acid when compared to platelets from normal rabbits.

In the many animal studies reported, few researchers have considered the possible importance of a raised blood cholesterol level which may lead to a raised cholesterol content in the blood platelets in these animals. Investigations into the release of arachidonic acid from platelet phospholipids have not previously been carried out. The present chapter therefore reports experiments carried out on platelets from

rabbits fed either a cholesterol supplemented diet or a normal diet, in order to answer some of the problems discussed above. These were:

1. Whether hypercholesterolaemia in rabbits gives rise to platelets enriched with cholesterol.

2. Whether platelets from hypercholesterolaemic rabbits show altered phospholipase A_2 activity.

3. Whether these platelets can synthesise more thromboxane A_2 than platelets from rabbits fed a normal diet.

6.2 Summary of Investigations

These experiments were carried out in the laboratory of Dr. M. Johnson at ICI Pharmaceuticals Division. Lop-eared rabbits weighing approximately 3 kg were divided into two groups. One group of 3 rabbits were fed a normal diet, while another group of 9 rabbits were fed the same diet supplemented with 0.5 per cent cholesterol. The dietary regimen was maintained for 35 days.

1. Blood samples were taken once a week from each rabbit to assay the serum cholesterol content.

2. After 27-29 days, blood samples were taken from rabbits for the following investigations:

a) Platelets were analysed for cholesterol and phospholipid content;

b) Aggregation was tested in PRP samples;

c) Platelet MDA production was assayed by the method of Stuart et al. (1975);

d) Thromboxane A_2 production by platelets was assayed by bioassay.

Rabbits were used for this cholesterol feeding experiment because hypercholesterolaemia was easily induced in these animals by dietary means. Rats were found not to be suitable for cholesterol feeding experiments. Platelets isolated from rats fed a diet supplemented with 1 per cent cholesterol were found to have a cholesterol:phospholipid molar ratio which was the same as platelets from rats fed a normal diet.

6.3 Serum Cholesterol and Platelet Analysis

Blood was taken weekly and assayed for serum cholesterol content by Mrs. S. Jones. Blood samples (1 ml) were allowed to clot and serum cholesterol was determined by an automated technique using the cholesterol oxidase assay method as described in Chapter 2. Table 6.1 shows that there was a significant difference in serum cholesterol levels between control and cholesterol-fed rabbits within 7 days of commencement of diet.

Samples of platelets were prepared for analysis after 27-29 days of the dietary regimen. On the 27th, 28th and 29th day, 50 ml of blood was taken from 1 control and 3 cholesterol fed rabbits for preparation of platelets for investigation. Samples of platelets were taken from the same blood sample for analysis of cholesterol and phospholipid. Table 6.2 shows that the cholesterol:phospholipid molar ratio was significantly higher in platelets from the cholesterol-fed group of rabbits. This result is in agreement with the results of Yoshida et al. (1977) in cholesterol-fed guinea-pigs.

6.4 Aggregation Studies

Samples of platelets in PRP were prepared from blood taken from each animal and were tested for aggregation in response to collagen. The results showed great variation of response in samples from different rabbits within the same group, and so no difference in either the extent or rate of aggregation of platelets from hypercholesterolaemic and normal rabbits could be detected. Zmuda et al. (1977) had reported enhanced sensitivity of PRP samples from atherosclerotic rabbits to ADP. Thus enhanced sensitivity of PRP samples from cholesterol-fed rabbits might have been expected in these experiments. Joist et al. (1976) showed that platelets from hypercholesterolaemic rabbits when resuspended in Tyrode's albumin buffer showed enhanced aggregation to collagen. It was possible therefore that the altered plasma lipid environment may

Table 6.1. Time course of serum cholesterol levels in rabbits during dietary regimen

Duration of diet (days)	Control diet (n=3)	Cholesterol-enriched diet (n=9)	P <
0	26.7±2.9	25.8±2.63	N.S.
7	26.3±1.5	174 ± 35	0.05
14	35.0±4.0	314 ± 52	0.02
21	31.0±4.0	405 ± 72	0.02
27-29	34.0±4.7	488 ±104	0.05
35	26.0±8.5	835 ± 97	0.001

All results are shown ± S.E.M. p values were calculated using students t-test.

Table 6.2. Analysis of rabbit platelets after 27-29 days of dietary regimen

	Control diet (n = 3)	Cholesterol diet (n = 9)	P <
Cholesterol phospholipid molar ratio	0.621±0.047	0.755±0.028	0.05

Cholesterol and phospholipid were assayed by the standard techniques as described in Chapter 2.

have interfered with the mechanism by which collagen stimulated platelets to aggregate in rabbit PRP thereby masking any difference in aggregability, and this effect may be absent in resuspended rabbit platelets.

6.5 Phospholipase A_2 Assays

Phospholipase A_2 activity was assayed using the technique described in Chapter 4 for once resuspended cells to determine whether the activity of this enzyme was altered in platelets from hypercholesterolaemic rabbits. It was shown previously that phospholipase A_2 activity could be detected in once resuspended rat platelets (Chapter 4), but that no difference in activity could be detected when platelets were enriched with cholesterol by the *in vitro* method compared to control platelets (Chapter 5). In these rat platelet phospholipase A_2 studies (Chapter 5), [1-^{14}C] oleoyl PC was used as the phospholipase A_2 substrate. In the present rabbit platelet study [1-^{14}C] arachidonyl PC was used because it was considered possible that [1-^{14}C] oleoyl PC may not be a suitable substrate to use to show a subtle difference in phospholipase A_2 activities.

[1-^{14}C] arachidonoyl PC would not be available to phospholipase C catalysed hydrolysis as the substrate for this enzyme is thought to be specifically phosphatidylinositol (Lapetina et al., 1980). Further metabolism of arachidonic acid by the platelets was restricted by the fatty acid free albumin incorporated in the resuspending buffer which was included to sequester the released fatty acid.

Indomethacin was used in the phospholipase A_2 assays to inhibit cyclo-oxygenase activity which may metabolise the released arachidonic acid. Jesse and Franson (1979) showed that partially purified platelet phospholipase A_2 activity was 50 per cent inhibited in the presence of 75 μM indomethacin. 10 μM indomethacin was used in the present experiments, a concentration where inhibition of phospholipase A_2

activity was minimal (see Jesse and Franson, 1979). Siegel et al. (1979) have shown that indomethacin can affect platelet lipoxygenase activity, in that arachidonic acid could be directed toward this pathway away from the cyclo-oxygenase pathway. However, at a concentration of 10 μM indomethacin, the results of Siegel et al. (1979) indicate little or no change in lipoxygenase activity, whereas at 100 μM indomethacin, there may be a considerable increase in lipoxygenase activity. Blackwell et al. (1977) had reported previously that indomethacin did not inhibit phospholipase A_2 activity at a concentration of 100 μM indomethacin in platelet homogenates. Indomethacin at a concentration of 10 μM used in the present work was therefore considered unlikely to interfere with phospholipase A_2 activity.

Phospholipase A_2 activity was assayed in platelet samples prepared from rabbits after 27-29 days on the dietary regimen. These were either stimulated with collagen or unstimulated (see Table 6.3). Platelet concentrations were adjusted so that the platelet counts were approximately the same in the two groups tested. The results show that there was a significantly enhanced phospholipase A_2 activity on stimulation with collagen in platelets from cholesterol-fed rabbits compared to platelets from normal rabbits. There was no difference in the activity of this enzyme between the two groups in unstimulated platelets. The results suggest therefore that an enhanced phospholipase A_2 activity was detectable only when the platelets were stimulated. The background unstimulated activity may be slightly higher in cholesterol-fed rabbit platelets, but the difference was not significant in these studies.

Table 6.4 shows results for a further phospholipase A_2 assay which was carried out on the 35th day of the dietary regimen. Platelet samples were prepared from the 6 cholesterol fed rabbits which had the highest

Table 6.3. Phospholipase A_2 activity of rabbit platelets after 27-29 days of dietary regimens

	Phospholipase A_2 activity	
	Unstimulated % conversion	Stimulated (2 µg/ml collagen)
Control diet (n = 3)	0.27 ± 0.23	0.10 ± 0.021
Cholesterol diet (n = 9)	1.14 ± 0.50	1.26 ± 0.256
P <	N.S.	0.05

Phospholipase A_2 assays were carried out on platelets resuspended once in modified Tyrode's buffer containing fatty acid free albumin (3.5 mg/ml), glucose (5 mM), [Ca^{2+}] (0.2 mM) pH 7.4. [1-^{14}C] arachidonoyl PC was added to 0.5 ml platelet sample and stirred in the aggregometer. After 1 min, collagen was added to stimulate the platelets, or buffer was added. The incubation was terminated after 10 min as described in Chapter 4. Phospholipase A_2 activity is expressed as per cent conversion of [1-^{14}C] arachidonyl PC to [1-^{14}C] arachidonic acid. All platelet samples of 0.5 ml contained 2.2×10^8 platelets.

Table 6.4. Phospholipase A_2 activity of rabbit platelets after 35 days of dietary regimen

	Phospholipase A_2 activity % conversion stimulated
Control diet (n = 3)	1.43 ± 0.59
Cholesterol diet (n = 6)	1.60 ± 0.27
P <	N.S.

Platelet samples (0.5 ml) were stimulated with 4 μg/ml collagen $[Ca^{2+}] = 0.4$ mM.

Control platelet samples contained 1.58×10^8 platelets/0.5 ml. Platelet samples from cholesterol-fed rabbits contained 1.66×10^8 platelets/0.5 ml.

Legend as for Table 6.3.

serum cholesterol levels, and from the 3 control rabbits. Higher concentrations of Ca^{2+} and collagen were used in an attempt to stimulate the phospholipase A_2 activity to show greater conversion of substrate to product, and to increase the difference in activities observed in the previous assay. No significant difference was detected between the two groups, but the activities, given as per cent conversion, were increased slightly in both groups compared to the assays carried out during days 27-29. The low activity suggests that there may be restriction of availability of substrate to the enzyme.

In the phospholipase A_2 assay carried out on the 35th day, it was observed that in 3 of the 6 samples of platelets from cholesterol-fed rabbits tested, aggregation of the platelets took place on addition of collagen even though indomethacin was present. This did not occur in any of the control samples. This suggests the possibility that some arachidonic acid was available for further metabolism to prostaglandins and thromboxanes, but this would not be expected to give artificially low conversions of substrate to product. Metabolites of arachidonic acid would be located in the fatty acid peak in the tlc system used for separation of [1-^{14}C] arachidonoyl PC and [1-^{14}C] arachidonic acid.

6.6 Metabolism by Platelets of Exogenous Arachidonic Acid

These assays were performed on platelet samples prepared from blood on the 27th-29th day of the dietary regimen. Platelet samples were tested for arachidonic acid metabolism in two ways:

1. MDA production was assayed by the method of Stuart et al. (1975).

2. Thromboxane A_2 production was assayed by bioassay by the method described in Chapter 2. These two assays were carried out by Dr. M. Johnson.

Table 6.5. Thromboxane A_2 assay of rabbit platelets after 27-29 days of dietary regimen

	Thromboxane A_2 production ng/ml PRP
Control diet (n = 3)	0.8 ± 0.2
Cholesterol diet (n = 9)	4.4 ± 0.6
P <	0.05

Thromboxane A_2 production was induced in PRP samples by the addition of arachidonic acid (3.0×10^{-4}M).

For details of method see Chapter 2.

Table 6.6. MDA production by rabbit platelets after 27-29 days of dietary regimen

	Unstimulated	Stimulated
	(nmol/10^9 platelets)	
Control diet (n = 3)	1.72 ± 0.19	6.42 ± 0.326
Cholesterol diet (n = 9)	2.37 ± 0.23	8.15 ± 0.579
P <	0.05	0.05

Stimulation of the samples was by the addition of arachidonic acid (1×10^{-3}M).

The results obtained from these assays are shown in Tables 6.5 and 6.6 respectively, and clearly indicate that the arachidonic acid metabolic pathway was more active in the platelets from the cholesterol-fed rabbits. MDA production is considered to be a good index of the activity of the prostaglandin synthesising pathway in platelets (Smith et al., 1976). It has been reported to be produced by platelets during secondary aggregation (Macfarlane et al., 1977) and MDA formation is catalysed by the activity of thromboxane synthase (McMillan et al., 1978).

6.7 Discussion

The results from the present investigation showed that:

1. Rabbits fed a 0.5 per cent cholesterol supplemented diet developed significantly increased serum cholesterol levels.

2. Platelets from the hypercholesterolaemic rabbits had a raised cholesterol:phospholipid molar ratio. The ratio was 21.6 per cent higher than in platelets from rabbits fed a normal diet.

3. Platelets from the hypercholesterolaemic rabbits showed enhanced phospholipase A_2 activity under certain specific conditions.

4. Platelets from the hypercholesterolaemic rabbits showed enhanced metabolism of exogenously added arachidonic acid to thromboxane A_2 and MDA compared to platelets from rabbits fed a normal diet.

The phospholipase A_2 activity was significantly increased in platelets from hypercholesterolaemic rabbits when stimulated with collagen at a final concentration of 2 μg/ml in the presence of 0.2 mM Ca^{2+}. In unstimulated platelets there was no significant difference in phospholipase A_2 activities. Phospholipase A_2 assays were repeated on the 35th day of the dietary regimen under different conditions (Table 6.4). Collagen was added to a final concentration of 4 μg/ml in the presence of 0.4 mM Ca^{2+}. The phospholipase A_2 activities were stimulated to the same extent in both the normal and cholesterol-rich platelets.

This suggests that the conditions used in the latter experiment stimulated the enzyme superoptimally, thus obscuring any subtle differences.

Since this investigation was carried out, a similar study has been reported by Kawaguchi et al. (1981). These workers fed rabbits a diet supplemented with 1 per cent cholesterol for 3 months. The investigations carried out on platelets from these animals included 1) assay of thromboxane B_2 production 2) assay of phospholipase A_2 activity 3) assay of phospholipase C and diacylglycerol lipase activities and 4) assay of cholesteryl esterase activity. Some of the results from the study by Kawaguchi et al. confirm some of the findings reported in this chapter. These include the increased production of thromboxanes from arachidonic acid by platelets from hypercholesterolaemic rabbits compared to those from control rabbits. Also, the cholesterol fed rabbits showed a markedly increased plasma cholesterol level (745 mg/100 ml) compared to control rabbits (78 mg/100 ml blood). Kawaguchi et al. assayed for the cholesterol content of platelets, and showed that platelets from the hypercholesterolaemic rabbits contained 3 times as much cholesterol as normal control platelets. These workers did not specify whether this result was for total cholesterol or unesterified cholesterol, although it can be assumed that these results relate to total cholesterol. Kawaguchi et al. therefore did not report whether platelet membrane unesterified cholesterol content was altered; a result which would have been of interest to the present study. As mentioned above, Kawaguchi et al. assayed for cholesteryl esterase activity and also for cholesterol hydrolase activity. They showed that esterification of cholesterol was enhanced, and hydrolysis of cholesterol esters was reduced in platelets from hypercholesterolaemic rabbits compared to platelets from control rabbits. These results probably explain the finding of increased total cholesterol content in these platelets.

Kawaguchi et al. carried out assays to determine the activity of enzymes catalysing the hydrolysis of platelet membrane phospholipids to release arachidonic acid. Their results showed that there were no differences in either phospholipase A_2 activity, or phospholipase C/ diacylglycerol lipase activities between platelets from normal and hypercholesterolaemic rabbits. In the present study reported in this chapter, a significant increase in phospholipase A_2 activity was observed in cholesterol rich rabbit platelets and it is therefore necessary to review and compare the technique used for the assay of this enzyme described in this chapter and the technique described by Kawaguchi et al.

In the phospholipase A_2 assay described by Kawaguchi et al., one of two substrates was used. This was either [1-^{14}C] arachidonyl PC or platelets prelabelled with [1-^{14}C] arachidonic acid. The prelabelling was achieved by a combination of the methods described by Rittenhouse-Simmons et al. (1976) and Bills et al. (1976). When [1-^{14}C] arachidonyl PC was used in the incubation system, carrier PC was also included. Buffer was added to these dry lipids and the mixture sonicated. The reaction was started by the addition of sonicated platelets. The final mixture (1 ml) contained 0.05M glycine-NaOH buffer (pH 9.5), 10 mM $CaCl_2$, 200 nmol of substrate and carrier PC, and 1 mg of protein from sonicated platelets. Where assays were carried out using labelled platelets as substrate, 10 µg of adrenaline was added to the incubation system.

In their assays using [1-^{14}C] arachidonyl PC as substrate for phospholipase A_2, Kawaguchi et al. showed that the per cent release of radioactivity (due to [1-^{14}C] arachidonate) in the control and hypercholesterolaemic rabbit platelets after 10 min was 22.6 and 22.9 respectively, and after 20 min was 36.0 and 35.0 respectively. These results indicated that there was no difference in phospholipase A_2

activity between platelets from the two groups. The conditions used in the assay by Kawaguchi et al. described above are different in many respects from the conditions used in the assays described and used in this thesis. Kawaguchi et al. used a pH of 9.5 as opposed to 7.4 in the present project. This high pH is known to be optimal for phospholipase A_2 activity (Billah et al., 1980), and would possibly stimulate the enzyme. The concentration of Ca^{2+} used by Kawaguchi et al. was 10 mM, another factor likely to stimulate the enzyme. The effect of a large quantity of substrate used by Kawaguchi (approximately 1.5 mg of exogenous phospholipid added to 1 mg of platelet protein as opposed to approximately 8 μg per mg used in the present project) on the activity of the enzyme is unknown. It could be that so much substrate enhances the activity of the enzyme. In the assays carried out by Kawaguchi et al. using [1-^{14}C] arachidonyl PC as substrate, it is very likely that the phospholipase A_2 was stimulated super-optimally, thereby obscuring any subtle differences in activity between different samples. The results presented from the work carried out in this chapter using slightly different conditions to assay for phospholipase A_2 activity strongly suggests that differences in activity of this enzyme may only be observed under very specific conditions (see Tables 6.4 and 6.5). Kawaguchi et al. stated that when platelets prelabelled with [1-^{14}C] arachidonic acid were used as substrate for the phospholipase A_2 assay, "the total amount of radioactive arachidonic acid released showed almost no change between control and hypercholesterolaemic platelets". The data these workers presented does not support this statement. Firstly, the proportion of the total [1-^{14}C] arachidonic acid incorporated into the phospholipids PC and PI were different in the two kinds of platelets. Of the radioactivity incorporated, 47.2 per cent was located in PC in normal platelets,

whereas 61.7 per cent was located in PC in hypercholesterolaemic platelets. Also, incorporation of radioactivity into PI was 17.5 per cent and 7.5 per cent respectively. Secondly, after incubation of prelabelled platelets with adrenaline for the assay of phospholipase A_2 activity, the radioactivity located in PC was 17.9 and 26.1 per cent, and the radioactivity in PI was 16.2 per cent and 5.2 per cent in control and hypercholesterolaemic platelets respectively.

These results suggest that the extent of incorporation of [1-^{14}C] arachidonic acid into these two phospholipids is different in the two types of platelets. By calculation, the proportion of [1-^{14}C] arachidonic acid released from PC was 29.3 per cent in control platelets, and 35.6 per cent in hypercholesterolaemic platelets. Also, the proportion released from PI was 1.3 and 2.3 per cent respectively. It remains unknown whether these differences were significant but clearly it is extremely difficult to compare phospholipase A_2 activities in prelabelled platelets when the rate of incorporation of the fatty acid is different between the two types of platelets.

In view of the results presented in this chapter, that hypercholesterolaemic rabbit platelets showed an increased cholesterol: phospholipid molar ratio and enhanced phospholipase A_2 activity under specific conditions, experiments were carried out on human platelets (Chapter 7). This was carried out to determine whether platelets from hypercholesterolaemic subjects had a raised cholesterol content, and whether these platelets showed enhanced phospholipase A_2 activity. Phospholipase A_2 assays were carried out on crude human platelet membrane fractions (Chapter 7) and rat platelet membrane fractions (Chapter 8), because it was shown that the per cent conversion of substrate to product could readily be detected (Chapter 4) in membrane preparations. It was preferred that the per cent conversion should be higher than detected with resuspended rabbit platelets as reported in this chapter.

Chapter 7

Investigation of Human Platelets

7.1 Introduction

Cooper (1969) studied the sera and red cells of 3 patients with severe liver disease. He described the red cells as "spur cells". The term "spur cell" has been applied to these bizarre red cells to describe their unusual thorny projections and to distinguish them from the morphologically similar but chemically dissimilar acanthocytes seen in patients with abetalipoproteinaemia (Ways et al., 1963). Cooper (1969) observed that the unesterified cholesterol content of spur cells was on average 52 per cent higher than in normal red cells from normal subjects. He reported that the cholesterol content of normal red cells incubated in spur cell serum increased and they took on a spur cell like appearance. Conversely spur cells incubated in normal serum lost cholesterol and took on a normal red cell appearance. It was apparent that red cells served as a repository for cholesterol which was at a high concentration in the blood in these subjects. Cooper suggested that the cholesterol taken up by the red cells was initially loosely bound to serum lipoproteins.

In a further investigation, Cooper et al. (1972) carried out an analysis of lipoproteins and red cell membranes from patients with liver disease. They showed that there was no correlation between serum cholesterol concentration and red cell content of free cholesterol. However they observed a close relationship between red cell and serum (or isolated LDL) free cholesterol content when each was considered relative to phospholipid. Spur cells were considered to be red cells which took up cholesterol in amounts relative to the cholesterol: phospholipid molar ratio of the serum.

Cooper et al. (1975) undertook to establish an artificial system that reproduced the cholesterol disproportion in the serum of patients with spur cells in order to study this factor in the absence of other

possible influencing factors. These workers prepared cholesterol-phospholipid dispersions (as discussed in Chapters 1 and 3). They showed that when normal red cells were incubated with cholesterol-rich dispersions they developed spur cell-like morphology concomitant with becoming enriched with cholesterol. Cooper et al. also showed in the same study that incubation of spur cells in a cholesterol-depleted medium gave rise to red blood cells which were morphologically normal and which had a normal cholesterol content. In a more recent study, Cooper et al. (1978) showed that the membrane fluidity of red cells was decreased as a result of cholesterol enrichment by this in vitro technique.

Human subjects with familial hyperbetalipoproteinaemia (type lla) are at higher risk than normal subjects to severe early-age atherosclerosis (Lees et al., 1973). Type lla subjects are recognised as having an elevated blood cholesterol level. These subjects may have xanthomatosis (the appearance of fat-containing lesions in the skin and subcutaneous tissues) and this often occurs in the Achilles tendon region. Grayish opacification may be observed at the corneal limbus in young adult type lla subjects. This is described as arcus corneae (Lees et al., 1973). Both altered platelet function and a high blood cholesterol level have been implicated as factors in the pathogenesis of atherosclerosis and thrombosis (Davignon, 1978; Steinberg, 1978). It was shown that platelets from type lla subjects were more sensitive to the aggregating agents adrenaline, ADP and collagen when compared to platelets from normal subjects (Carvalho et al., 1974a; Shattil et al., 1977). It appeared from the latter study that there was a good correlation between the proportion of cholesterol to phospholipid in the serum LDL and in the platelets in the subjects examined. However the cholesterol to phospholipid molar ratio was not altered in red cells in the type lla

subjects of the study by Shattil et al. (1977) and this suggested that a simple cause and effect relationship did not appear to exist for the red cell cholesterol content. It has been reported that platelets from type IIa subjects were capable of synthesising greater quantities of thromboxane A_2 than normal platelets (Colman, 1978; Tremoli et al., 1979a), and of showing enhanced platelet factor 3 availability (Nordoy and Rodset, 1971). In the studies carried out by Tremoli et al. (1979a) and by Nordoy and Rodset (1971) raised plasma cholesterol levels were reported in the type IIa subjects examined. However, in neither of these studies were platelet cholesterol levels determined, therefore it remained unknown whether a raised platelet cholesterol to phospholipid molar ratio was in part responsible for the observed hyperactivity of platelets from type IIa subjects.

In a recent study, Shastri et al. (1980) confirmed that platelets from type IIa subjects were hyperactive to the aggregating agents adrenaline, ADP and collagen. These subjects had a raised plasma cholesterol level compared to control subjects, but they reported that platelet cholesterol content was not significantly higher than that found in normal platelets. It was not clear from the study by Shastri et al. (1980) whether the proportion of cholesterol to phospholipid was elevated in the plasma of the type IIa subjects examined which, as discussed above, may be an important factor in influencing the cholesterol:phospholipid molar ratio in the platelets.

Most recently, Owen et al. (1981) studied the lipid composition and aggregation of platelets from human subjects with liver disease. These workers showed that the cholesterol:phospholipid molar ratio was raised by 13 per cent in the platelets from these patients compared to platelets from normal healthy subjects. The cholesterol content was also raised in erythrocytes from the subjects with liver disease.

No plasma cholesterol determinations were reported from this study by Owen et al. (1981), but the subjects with liver disease may have had high blood cholesterol levels and whether this would have influenced the cholesterol content of platelets and erythrocytes remains unknown. These workers carried out aggregation studies and showed that platelets from subjects with liver disease (with raised cholesterol content) were not more sensitive than normal platelets and in some cases were less sensitive. Platelets from patients were found to contain proportionately less arachidonic acid and the phosphatidylcholine: sphingomyelin ratio was raised compared to normal platelets. These two factors may have influenced platelet aggregating ability, and any hypersensitivity due to increased cholesterol content may have been masked by these and other differences in platelet composition.

As has been discussed in Chapter 1, Shattil et al. (1975) showed that the proportion of cholesterol to phospholipid in platelets could be increased *in vitro*, and the result of this treatment was to induce hypersensitivity to ADP and adrenaline stimulated aggregation. Platelet membranes enriched with cholesterol in this way had increased microviscosity (Shattil and Cooper, 1976) and further investigations suggested the possibility that cAMP production in these platelets may be enhanced (Sinha et al., 1976; Insel et al., 1978).

In attempts to normalise the sensitivity of platelets from type IIa subjects, clofibrate administration to these subjects and the resultant effect on platelet function has been investigated by Carvalho et al. (1974b). These workers showed that clofibrate therapy resulted in platelets isolated from type IIa subjects requiring the same minimum concentration of adrenaline to give a full aggregation response as for normal control platelets. The effect of halofenate was also investigated. It was found to be more potent in its effect than clofibrate,

and normalised the sensitivity of platelets from type IIa subjects to the aggregating agents ADP, adrenaline and collagen (Colman et al., 1976). It was subsequently shown that halofenate therapy did not inhibit arachidonic acid induced aggregation or MDA production by platelets from type IIa subjects (Favis and Colman, 1977). Thus halofenate possibly acted by inhibiting mechanisms involved in the release of arachidonic acid from platelet phospholipids. This suggests the possibility that increased thromboxane A_2 production by type IIa platelets may be due to an increased rate of hydrolysis of arachidonic acid from platelet membrane phospholipids, this reaction being considered by some researchers to be an important rate limiting step in the metabolism of arachidonic acid (Blackwell et al., 1977).

In view of the findings discussed above, and the observations that platelets from hypercholesterolaemic rabbits had an increased cholesterol:phospholipid molar ratio which showed enhanced phospholipase A_2 activity (Chapter 6), a study was undertaken to examine platelets isolated from human hyperlipidaemic subjects and were compared to platelets from normal subjects. This study was carried out in the laboratory of Dr. M. Johnson at ICI Pharmaceuticals Division in collaberation with Dr. M. Stone at the Leigh Health Centre. Before this trial was carried out, experiments were carried out in Edinburgh to alter the cholesterol content of human platelets by the <u>in vitro</u> method described by Shattil et al. (1975) (see Chapter 3).

7.2 <u>Cholesterol Enrichment and Depletion of Human Platelets In Vitro</u>

Human blood was kindly provided by donors at the Blood Transfusion Service (BTS), Royal Infirmary of Edinburgh. Units of blood were collected under standard BTS conditions. These units of blood were tested for hepatitis B antigen by Dr. R. Hopkins prior to any experimentation. PRP was generally prepared within 2-3 hours after

Table 7.1 Analysis of human platelets after in vitro alteration of cholesterol content

	Cholesterol: phospholipid molar ratio	Cholesterol: protein weight ratio	Phospholipid: protein weight ratio
Tyrode's incubated	0.595 ± 0.021	0.071 ± 0.003	0.250 ± 0.019
Cholesterol depleted	0.462 ± 0.016	0.053 ± 0.001	0.232 ± 0.012
Cholesterol normal	0.565 ± 0.027	0.063 ± 0.004	0.245 ± 0.01
Cholesterol enriched	0.656 ± 0.012	0.082 ± 0.003	0.250 ± 0.021

Samples of human PRP (5 ml) were incubated for 3 hrs at 37°C with an equal volume of either modified Tyrode's buffer, cholesterol-poor liposomes, cholesterol-normal liposomes, or cholesterol-rich liposomes prepared as described in Chapter 2.

Results show means $\pm$ S.E.M. of 4 experiments. Significance was calculated using students t-test. This showed that:-

1) The cholesterol:protein ratio in the cholesterol-depleted platelets was significantly reduced compared with Tyrode's incubated control platelets ($p < 0.01$), and significantly reduced when compared with control platelets incubated with cholesterol-normal liposomes ($p < 0.05$). Cholesterol enriched platelets showed a significantly increased cholesterol:protein ratio compared with Tyrode's incubated controls ($p < 0.05$) and control platelets incubated with cholesterol-normal liposomes ($p < 0.001$).

2) The cholesterol:phospholipid ratio was significantly reduced in cholesterol depleted platelets compared with Tyrode's incubated controls ($p < 0.01$) and liposome incubated control platelets ($p < 0.05$). Cholesterol enriched platelets showed a cholesterol:phospholipid ratio which was significantly increased compared to both liposome incubated control platelets ($p < 0.05$) and Tyrode's incubated control platelets ($p < 0.05$).

Figure 7.1. Aggregation of human platelets after 5 hrs incubation with liposomes to alter platelet cholesterol content

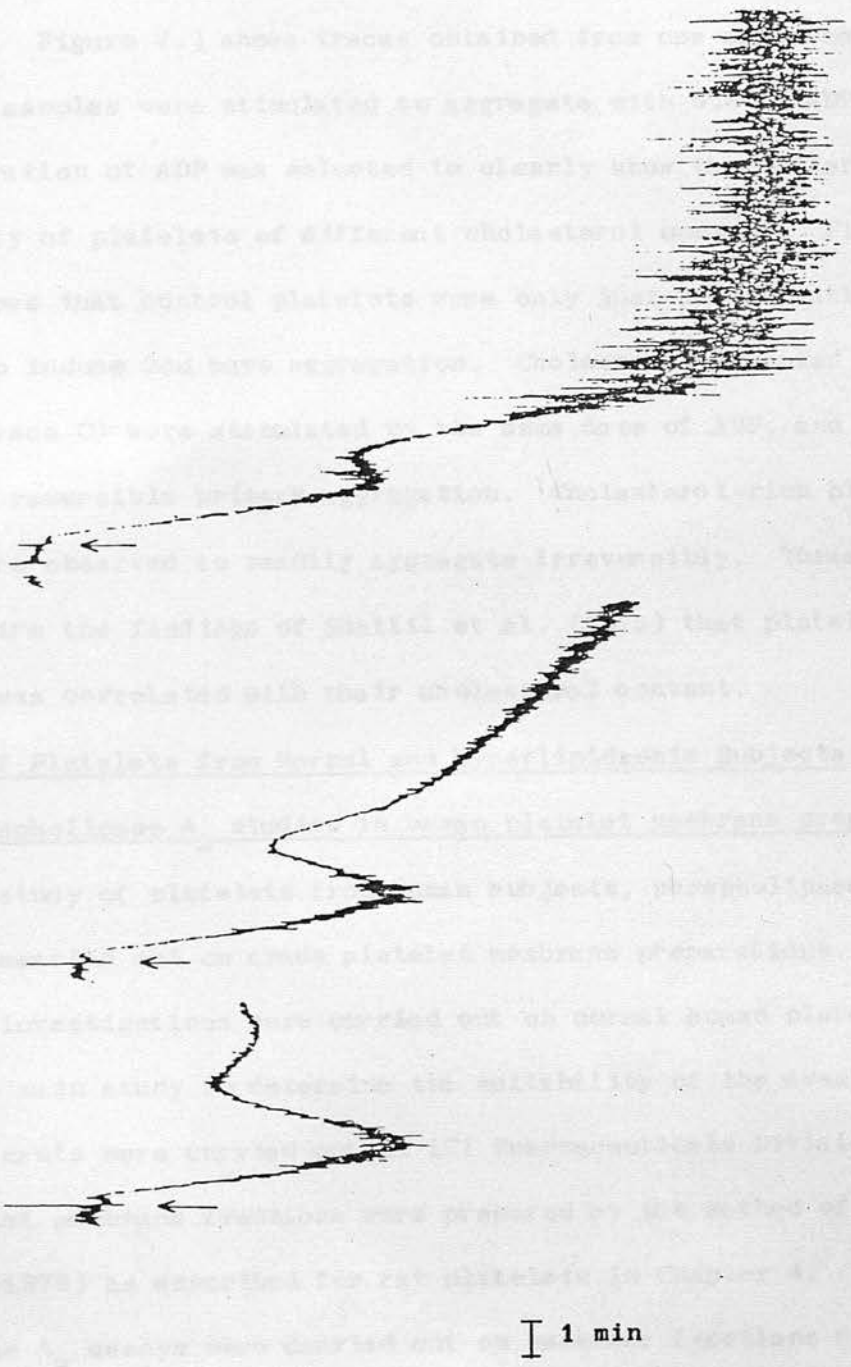
Traces show aggregation induced by ADP (0.2 μM).

Trace A shows irreversible aggregation of cholesterol-enriched platelets.

Trace B shows slow irreversible aggregation of control-(cholesterol-normal) platelets.

Trace C shows reversible aggregation of cholesterol-depleted platelets.

Figure 7.1.



blood collection, and samples were incubated with liposome suspensions to alter platelet cholesterol content as described for rat PRP in Chapter 3.

Table 7.1 shows mean results for analysis of human platelets from 3 experiments to deplete and increase the proportion of cholesterol to phospholipid. Figure 7.1 shows traces obtained from one experiment where PRP-liposome samples were stimulated to aggregate with 0.2 μM ADP. This concentration of ADP was selected to clearly show the differences in sensitivity of platelets of different cholesterol content. Figure 7.1 (trace B) shows that control platelets were only just sufficiently stimulated to induce 2nd wave aggregation. Cholesterol-depleted platelets (trace C) were stimulated by the same dose of ADP, and underwent rapid reversible primary aggregation. Cholesterol-rich platelets (trace A) were observed to readily aggregate irreversibly. These results confirm the findings of Shattil et al. (1975) that platelet sensitivity was correlated with their cholesterol content.

7.3 Study of Platelets from Normal and Hyperlipidaemic Subjects

1. Phospholipase A_2 studies in human platelet membrane preparations

In the study of platelets from human subjects, phospholipase A_2 assays were carried out on crude platelet membrane preparations. Two preliminary investigations were carried out on normal human platelets prior to the main study to determine the suitability of the assay. These experiments were carried out at ICI Pharmaceuticals Division. Human platelet membrane fractions were prepared by the method of Wong and Cheung (1979) as described for rat platelets in Chapter 4. Phospholipase A_2 assays were carried out on membrane fractions rather than resuspended platelets in view of the low activities found in preparations of resuspended rabbit platelets (Chapter 6). The substrate used for the assay was [1-^{14}C]oleoyl PC rather than [1-^{14}C] arachidonyl PC, in order to exclude the possibility of influence of

arachidonic acid metabolites on phospholipase A_2 activity. The incubation period used was 35 min at 37°C and 1 ml samples contained 300 µg of membrane protein, 2.0 mM Ca^{2+} and fatty acid free albumin (0.5 mg/ml) in tris HCl buffer pH 9.0. This incubation system was the same as described for use with rat platelet membranes in Chapter 4. An incubation period of 35 min was used to provide optimal conditions for detection of phospholipase A_2 activity.

In the first experiment, PRP samples were left to stand for 1½ hours at 20°C. The purpose of this was to simulate the treatment the samples would receive during the experimental procedure in the main study of platelets from hyperlipidaemic subjects. This was the period of time it was anticipated it would take after venepuncture before processing of samples for preparation of membrane fractions. In this first experiment, the phospholipase A_2 activity was very low. The mean conversion of [1-^{14}C] oleoyl PC to [1-^{14}C] oleate was less than 3 per cent in triplicate samples. In the second preliminary experiment, PRP was processed for preparation of membrane fractions within 30 min of venepuncture. In triplicate samples, the mean conversion was 7.5 per cent. It was clear from these results that membrane fractions prepared from human platelets contained less detectable phospholipase A_2 activity, and that this activity was very labile.

2. Experimental details and results of study of platelets from normal and hyperlipidaemic subjects

Subjects under the care of Dr. M. Stone were selected to take part in the study. Six control subjects were selected who had a history of low or normal blood cholesterol levels (below 200 mg/100 ml). Six hyperlipidaemic subjects were selected who had been diagnosed with either type IIa or type IIb hyperbetalipoproteinaemia. These subjects had histories of high blood cholesterol levels (above 500 mg/100 ml)

and high LDL levels (above 300 mg/100 ml). Three of these subjects had suffered cardiovascular complications during the previous 2 years.

Blood samples were taken from 3 normal (control) and three test subjects by Dr. M. Stone at the Leigh Health Centre on one day, and samples were taken from the remaining subjects on another day. The subjects had fasted overnight for at least 12 hours.

60 ml of blood was taken from each subject from the antecubital vein of one arm into 3.8 per cent sodium citrate for platelet samples to be examined at ICI Pharmaceuticals Division.

PRP samples were prepared from the 60 ml blood sample, and approximately 20 ml of PRP was obtained from each subject. These samples were then transferred to ICI Pharmaceuticals Division as quickly as possible for processing. Investigations commenced within approximately $2\frac{1}{2}$ hours after the blood samples were obtained. The tests carried out were:

1. Phospholipase A_2 assay (Table 7.3)
2. MDA assay (Table 7.3)
3. Aggregation of PRP samples (Table 7.4)
4. Analysis of cholesterol, phospholipid and protein of samples of platelet membrane prepared for the phospholipase A_2 assay (Table 7.2).

Membrane samples prepared for phospholipase A_2 assays were analysed for protein prior to the assay of phospholipase A_2 activity in order that samples could be prepared containing the same amounts of membrane protein. Aliquots of these membrane preparations were then stored at $-10^{\circ}C$ until the lipids were extracted by the method of Bligh and Dyer (1959) as described in Chapter 2 for analysis of cholesterol and phospholipid. MDA assays were carried out on resuspended platelet samples by Dr. M. Johnson by the method of Stuart et al. (1975).

In the experiments carried out in this study, the following observations were made:

Table 7.2. Analysis of human platelet membrane preparations

	Cholesterol: phospholipid molar ratio	Cholesterol: protein weight ratio	Phospholipid: protein weight ratio
Control group (n = 4)	0.568 ± 0.031	0.050 ± 0.005	0.178 ± 0.024
Hyperlipidaemic group (n = 6)	0.591 ± 0.020	0.059 ± 0.007	0.204 ± 0.026
p <	N.S.	N.S.	N.S.

Results are presented $\pm$ S.E.M.. p values were calculated using students t-test.

In the control group, 2 samples had insufficient lipid for accurate assay, hence results for the remaining 4 are included.

Table 7.3. Phospholipase A_2 assay of human platelet membrane preparations

	Phospholipase A_2 activity % conversion	MDA production nmol/10^9 platelets
Control group (n = 6)	0.93 ± 0.09	11.5 ± 2.04
Hyperlipidaemic group (n = 6)	1.00 ± 0.36	8.04 ± 1.15
P <	N.S.	N.S.

Phospholipase A_2 activity was assayed by determining % conversion of [1-^{14}C] oleoyl PC to [1-^{14}C] oleate in 35 min in 1 ml samples containing 0.3 mg membrane protein, Ca^{2+} (2.0 mM) albumin (0.5 mg/ml) in tris HCl buffer at pH 9.0.

MDA production was assayed in resuspended platelets induced by 1 mM arachidonic acid.

Table 7.4. Aggregation of human PRP samples

	Extent (% max)	Rate (% min^{-1})
Control group (n = 6)	61.8±6.54	20.0±1.97
Hyperlipidaemic group (n = 5)	65.7±2.75	17.4±2.05
p <	N.S.	N.S.

Samples of PRP (0.25 ml) were tested for aggregation induced by collagen (final concentration of 1.6 μg/ml). Samples of PRP from one hyperlipidaemic subject aggregated spontaneously on stirring in the aggregometer. This result is not included in the figures above.

1. There was no detectable difference in the cholesterol:phospholipid molar ratio in the membrane preparations from platelets of the two groups (Table 7.2).

2. Phospholipase A_2 activity and MDA production were not significantly different between the two groups (Table 7.3).

3. Sensitivity of PRP samples to the aggregating agent collagen was not significantly different. However, PRP from one hyperlipidaemic subject aggregated spontaneously on stirring in the aggregometer.

7.4 Discussion

In the present study, platelets from normal control subjects and from hyperlipidaemic subjects were compared. No differences in sensitivity to collagen induced aggregation, or in arachidonic acid induced MDA production were detected between the two groups. Phospholipase A_2 activity was very low in all platelet samples, and any small difference in activities there may have been were not detected. It is clear that phospholipase A_2 activity in human platelet membranes was very labile when compared to similar studies carried out on rat platelets (see Chapters 4 and 8). It is difficult to conclude from the analysis results that the cholesterol content of platelets from these hyperlipidaemic subjects was not different from normal platelets because of the variation between samples from within the same group, and the low number of subjects in each group. This made statistical analysis unreliable and could only have been improved with a larger number of subjects in each group. The difference in cholesterol:phospholipid molar ratio of platelets from type IIa subjects compared to control platelets was reported by Colman (1978) to be 7 per cent. It is unlikely that a difference of this small amount would have been detected in the present study.

The experiments reported in Chapter 6 where platelets from hypercholesterolaemic rabbits were examined and compared with platelets from normal control rabbits had one major advantage over the present study with human subjects. This was that the two groups of rabbits studied were both very homogeneous groups each with very well defined characteristics. A common problem frequently encountered in human studies is the great variability between subjects within a given group. There are often differences in age, activities, diets and as was the case in the present study, the nature of the hyperlipidaemic condition could not be precisely defined. In respect of age, environment and activities the two groups of rabbits studied in Chapter 6 were very similar. The diet constituents were the same except for the one group taking the diet supplemented with cholesterol. Such well controlled conditions for human studies is often impossible and makes interpretation of results more difficult.

The results presented in this chapter reinforce the need for carefully controlled conditions for research of the type described here. It is clear that to investigate the effect of cholesterol enrichment on the platelet membrane, the *in vitro* technique described in Chapters 3 and 5 has several advantages. In the *in vitro* technique, it is only cholesterol which is altered, and no complications arise due to alteration of other platelet lipids such as described in the study of platelets from human subjects with liver disease by Owen et al. (1981).

In the final group of investigations described in the following chapter, rat platelets have been incubated *in vitro* to alter their cholesterol content, and also to enrich platelets with cholesterol analogues. Studies were carried out in the knowledge that essentially all conditions were well controlled and the cause and effect relationship between platelet sterol content and platelet function could be investigated.

Chapter 8

Effect of Alteration of Cholesterol Content and Cholesterol Analogue Enrichment on Rat Platelet Function

8.1 Introduction

It has been shown in the present project that rat platelets enriched with cholesterol by the *in vitro* method demonstrated enhanced sensitivity to collagen induced aggregation (Chapter 5). Human platelets which had been depleted of cholesterol by this method showed reduced sensitivity to ADP induced aggregation, and cholesterol-enriched platelets showed enhanced sensitivity to ADP induced aggregation compared to control platelets (Chapter 7). Also, it has been shown in the present project that platelets from hypercholesterolaemic rabbits were enriched with cholesterol, and under specific conditions showed enhanced phospholipase A_2 activity compared to platelets from normal rabbits.

It has been demonstrated that rat platelet membrane preparations provided a good source of phospholipase A_2 activity (Chapter 4). Details are reported in this chapter of investigations which were carried out to alter the rat platelet membrane cholesterol content by the *in vitro* technique (Chapter 3), and to study the phospholipase A_2 activity in membrane preparations. Much work has been carried out recently which demonstrated that sterols containing side chains of lengths different from that found in cholesterol dramatically altered the properties of artificial membranes (Suckling and Boyd, 1976; Craig, 1978; Suckling et al., 1979), as discussed in Chapter 1. The platelet membrane is a very important structure involved in the aggregation process. The platelet, when it is stimulated by a suitable agonist, swells to a spherical shape, and then pseudopodia form which are long extensions of membrane protruding out from the surface of the platelet. To investigate the importance of the precise dimensions of the cholesterol molecule in biological membranes, rat platelets were enriched with cholesterol analogues with side chains of different lengths.

8.2 Summary of Investigations of Rat Platelets

Results from 7 experiments are reported in which combinations of various investigations were carried out. Membrane dependent events, namely aggregation and phospholipase A_2 activity have been routinely studied except where otherwise stated. The importance of cholesterol for the normal function of rat platelets has been investigated by studying the effect of alteration of platelet cholesterol content. Further studies have been carried out to investigate the effect of incorporation of various cholesterol analogues into platelets. Cholesterol analogues were synthesised in this laboratory and kindly provided by Dr. I. Craig and Dr. K. Suckling.

1. By using the *in vitro* incubation method described by Shattil et al. (1975) and discussed in Chapter 3, cholesterol enrichment and depletion of rat platelets was carried out. The same technique was used to enrich rat platelets with the C_{21}-, C_{24}- and C_{26}-sterol analogues.

2. Analysis was routinely carried out to determine the sterol, phospholipid and protein content of crude platelet membrane fractions. These fractions were prepared for the phospholipase A_2 assays, and aliquots were retained for analysis.

3. Aggregation characteristics of the PRP-liposome mixtures were studied in the aggregometer when stimulated with collagen.

4. Scanning electron micrographs of platelet samples were kindly carried out by the Teaching and Research Centre, Western General Hospital, Edinburgh. Platelet samples were prepared after the incubation period and preserved in a glutaraldehyde fixative.

5. Platelet counts were carried out by the Coagulation Unit, Royal Infirmary of Edinburgh.

6. Phospholipase A_2 assays were carried out on crude platelet membrane preparations after the incubation period.

8.3 Alteration of Cholesterol Content of, and Incorporation of Cholesterol Analogues into Rat Platelets

In the present project, it has been shown that rat platelets could be enriched with or depleted of cholesterol. Also, it has been possible to enrich rat platelets with cholesterol analogues by incubating PRP with liposomes loaded with analogues. Preliminary investigations revealed that C_{21}-analogue enriched platelets demonstrated different characteristics compared to normal, cholesterol-depleted or cholesterol-enriched platelets. It was observed that in order to sediment C_{21}-analogue-enriched platelets, a prolonged period of centrifugation was required. Also, it was found that these platelets (in PRP-liposome mixtures) aggregated spontaneously in a cuvette on stirring the sample in the aggregometer. These observations suggested that the integrity of platelets was disrupted by incorporation of this cholesterol analogue.

Tables 8.1-8.8 show results for analysis of crude platelet membrane fractions. The results show that cholesterol depletion and enrichment of rat platelets occurred in each experiment, and the incorporation of the 3 cholesterol analogues was also successful.

Table 8.8 shows mean values for the cholesterol:phospholipid molar ratio and cholesterol:protein weight ratio calculated from the values in Tables 8.1-8.3, 8.5 and 8.6. The results show that the proportion of cholesterol to phospholipid and protein was significantly increased in cholesterol-enriched platelets compared to control platelets. Tables 8.2-8.4 show that after incubation for 3 hours, between 55 and 63 per cent of the total sterol present was the C_{21}-analogue in platelets incubated with C_{21}-analogue rich liposomes. Table 8.5 shows that the C_{24}-analogue was incorporated to a similar extent as the C_{21}-analogue, whereas in Table 8.6 it can be seen that incorporation of the C_{26}-analogue was much lower. In this experiment, only 25 per cent of the

Table 8.1. Cholesterol depletion and enrichment of rat platelets

	Analysis			Aggregation (% max. extent) to collagen (µg/ml)			Phospholipase A_2 (activity %)	P <
	C:PL	C:Pr	PL:Pr	4.0	5.6	8.0		
Cholesterol-depleted	0.523	0.054	0.200	67	65	66	8.14 ± 0.53	N.S.
Cholesterol-normal	0.577	0.060	0.204	0	0	69	4.64 ± 1.87	-
Cholesterol-enriched	0.734	0.076	0.216	22	65	64	14.9 ± 1.94	0.02

Rat PRP samples were incubated with liposome suspensions to deplete maintain or increase platelet cholesterol content (as described in Chapters 2 and 3).

After 3-3½ hr incubation, 0.25 ml PRP-liposome samples were tested for aggregation as described in Chapter 2. Crude membrane fractions were prepared from platelets as described in Chapter 2. Analysis was carried out on aliquots of crude membrane fractions as described in Chapter 2. Results for aggregation are for single tests only

C:PL = Cholesterol:phospholipid molar ratio
C:Pr = Cholesterol:protein weight ratio
PL:Pr = Phospholipid:Protein weight ratio

Phospholipase A_2 assays were carried out on crude membrane fractions. Crude platelet membrane suspensions (0.3 mg) were incubated at 37°C for 15 min in 1 ml buffer containing $CaCl_2$ (2 mM) albumin (0.5 mg/ml) tris (50 mM) pH 9.0. Reaction was started by addition of [1-^{14}C] oleoyl PC (10 nCi). Reaction was terminated by addition of 1 drop conc HCl, and lipids were extracted. Phospholipase A_2 activity is expressed as % conversion of [1-^{14}C] oleoyl PC to [1-^{14}C] oleic acid. Results given are means of 4 determinations $\pm$ S.E.M. Significance (p) was calculated from these results using students t-test for the enzyme assays,

Table 8.2. Cholesterol depletion and enrichment, and C_{21}-analogue enrichment of rat platelets

	Analysis			Aggregation (% max. extent) to collagen (µg/ml)		Phospholipase A_2	
	Sterol:PL	Sterol:Pr	PL:Pr	4.0	5.6	(activity %)	P <
C_{21}-analogue enriched	0.800 (55%)	0.055	0.159	-	-	4.45 ± 1.87	N.S.
Cholesterol-depleted	0.550	0.049	0.180	38	68	13.4 ± 7.9	N.S.
Cholesterol-normal	0.640	0.046	0.151	0	60	7.1 ± 2.66	-
Cholesterol-enriched	0.742	0.064	0.174	61	77	18.0 ± 3.11	0.05

Experimental details and abbrevations are as described in the legend to Table 8.1. In this experiment, a PRP sample was incubated with C_{21}-enriched liposomes. Hence analysis results show sterol:phospholipid molar ratios and sterol:protein weight ratios. The sterol:phospholipid result for C_{21}-analogue-enriched platelets shows the total sterol content, and the figure in parentheses gives the proportion of the total sterol which was present as the analogue. For analysis, the platelet lipid extract containing a mixture of C_{21}-analogue and cholesterol were quantitated by GLC as described in Chapter 2.
Results for aggregation are for single tests only. Phospholipase A_2 assay results shown means $\pm$ S.E.M. of 4 determinations.

Table 8.3. Cholesterol depletion and enrichment, and C_{21}-analogue enrichment of rat platelets

	Analysis			Aggregation (% max. extent) to collagen (µg/ml)	Phospholipase A_2	
	Sterol:PL	Sterol:Pr	PL:Pr	6.0	(activity %)	P <
Buffer incubated	0.62	0.051	0.166	49	28.9 ±3.93	0.02
C_{21}-analogue-enriched	0.804 (63%)	0.057	0.166	-	5.83±2.68	0.05
Cholesterol-depleted	0.465	0.044	0.189	55	23.9 ±1.76	0.01
Cholesterol-normal	0.555	0.056	0.216	65	14.3 ±1.22	-
Cholesterol-enriched	0.766	0.063	0.166	67	33.3 ±0.78	0.001

Experimental details and abbrevations are as described in legends to Tables 8.1 and 8.2. In this experiment, a PRP sample was incubated in an equal volume of modified Tyrode's buffer containing no liposomes.

Results for aggregation shown are for single tests only. Results shown for phospholipase A_2 assays show means ± S.E.M. of 4 determinations.

Table 8.4. C_{21}-analogue enrichment of rat platelets

	Analysis			Aggregation (% max. extent) to collagen (µg/ml)		Phospholipase A_2
	Sterol:PL	Sterol:Pr	PL:Pr	2	4	(activity %)
Buffer incubated	0.66	0.059	0.180	34	58	19.5
C_{21}-analogue-enriched	1.09 (59%)	0.098	0.210	-	-	8.3
Cholesterol normal	0.67	0.062	0.186	38	61	6.3

Experimental details and abbreviations are as described in the legends to Tables 8.1-8.3. In this experiment phospholipase A_2 results are the means of two determinations. Students t-tests were not calculated.

Table 8.5. Cholesterol enrichment and C_{24}-enrichment of rat platelets

	Analysis			Aggregation (% max. extent) to collagen (µg/ml)			Phospholipase A_2 (activity %)	P <
	Sterol:PL	Sterol:Pr	PL:Pr	1.6	2.0	2.4		
C_{24}-analogue-enriched	0.782 (61%)	0.068	0.163	-	-	-	10.7 ± 1.69	N.S.
Cholesterol-normal	0.646	0.050	0.150	0	5	62	6.9 ± 0.27	-
Cholesterol-enriched	0.704	0.060	0.162	36	42	74	12.5 ± 1.60	0.05

Experimental details and abbreviations are as described in the legends to Tables 8.1 and 8.2. In this experiment, a sample of PRP was incubated with C_{24}-analogue-rich liposomes. Results of aggregation are for single tests. Phospholipase A_2 assays were carried out in triplicate.
Aggregation traces for cholesterol-normal and cholesterol-enriched PRP-liposome samples stimulated by 2.4 ug/ml collagen are shown in traces A and B respectively in Figure 8.1. The aggregation trace of C_{24}-analogue-enriched platelets which occurred spontaneously without the addition of collagen is shown as trace D in Figure 8.1.

Scanning electron micrographs are shown of cholesterol-normal PRP-liposome sample (Plate 8.6) and C_{24}-analogue-enriched PRP-liposome sample (Plate 8.7).

Table 8.6. Cholesterol and C_{26}-analogue enrichment of rat platelets

	Analysis			Aggregation (% max. extent) to collagen (2.0 µg/ml) after incubation for:		
	Sterol:PL	Sterol:Pr	PL:Pr	2 hrs	3½ hrs	6 hrs
C_{26}-analogue-enriched	0.684 (25%)	0.070	0.184	64	64	67
Cholesterol-normal	0.600	0.051	0.170	44	51	45
Cholesterol-enriched	0.632	0.055	0.164	56	56	58

Experimental details and abbreviations are as described in the legends to Tables 8.1 and 8.2. In this experiment a sample of PRP was incubated with C_{26}-analogue-rich liposomes. Aggregation was tested after 2, 3½ and 6 hrs incubation of PRP with liposome suspensions. The aggregation traces obtained after 6 hrs incubation are shown in Figure 1.2.

Analysis was carried out on whole platelets in this experiment.

Table 8.7. Enrichment of rat platelets with cholesterol *in vitro*

	Cholesterol:phospholipid molar ratio	Cholesterol:protein weight ratio
Control (n = 5)	0.604 ± 0.018	0.053 ± 0.002
Cholesterol-enriched (n = 5)	0.716 ± 0.023	0.064 ± 0.003
P <	0.01	0.05

Results show means calculated from values obtained from Tables 8.1-8.3, 8.5 and 8.6.

Results include S.E.M.s and significance was calculated using students t-test.

Table 8.8. Cholesterol depletion and enrichment, and C_{21}-analogue enrichment of rat platelets

	Analysis		
	Sterol:PL	Sterol:Pr	PL:Pr
C_{21}-analogue-enriched	0.903 (83%)	0.094	0.210
Cholesterol-depleted	0.560	0.051	0.176
Cholesterol-normal	0.650	0.055	0.169
Cholesterol-enriched	0.700	0.060	0.171

Abbreviations are as described in legends of Tables 8.1 and 8.2.

Rat PRP samples were incubated with liposome suspensions for 5 hr and then analysed, or prepared for scanning electron micrographs. C_{21}-analogue-enriched samples are shown in Plate 8.2, cholesterol-depleted in Plate 8.3, cholesterol-normal in Plate 8.4 and cholesterol-rich in Plate 8.5.

Analysis was carried out on whole platelets in this experiment.

total sterol present was the C_{26}-analogue. Analysis of platelet lipid extracts which contained a mixture of cholesterol and analogue was carried out by gas liquid chromatography as described in Chapter 2.

In view of the observation that C_{21}-analogue incorporation into rat platelets dramatically altered their properties, further investigations were carried out. These investigations included aggregation, scanning electron microscopy and phospholipase A_2 assays.

8.4 Aggregation Studies

PRP-liposome samples were tested with various concentrations of collagen in order to identify submaximal concentrations which showed differences in sensitivity of the various samples. A supramaximal dose of collagen would induce maximum aggregation in all platelet samples masking small differences in sensitivities which may exist between the different groups. A concentration of collagen which was too low would not induce aggregation in any of the samples. Hence it was important to identify the concentration of collagen which would show small differences in sensitivity between different platelet samples.
The aggregation results showed that rat platelets enriched with cholesterol were more sensitive to collagen induced aggregation than control platelets (Tables 8.1-8.3, 8.5 and 8.6). These results agree with those presented in Chapter 5. In one experiment no difference was seen in sensitivity to collagen between normal and cholesterol-rich platelets. This was most likely due to the amount of collagen used being a supermaximal concentration. In three experiments, rat PRP was incubated with cholesterol-poor liposomes, and the platelets were depleted of cholesterol (Tables 8.1-8.3, 8.8). In three of these experiments, PRP-liposome samples were tested for aggregation with collagen (Tables 8.1-8.3). In two experiments, cholesterol-depleted rat platelets were more sensitive to collagen induced aggregation than

control platelets (Tables 8.1, 8.2). In the other experiment (Table 8.3), the extent of aggregation was very slightly less than control samples. When ADP (0.2 μM) was used to stimulate rat PRP-liposome samples, cholesterol-enriched platelets were more sensitive to aggregation than control platelets. At the same concentration of ADP, cholesterol-depleted platelets aggregated irreversibly. Hence cholesterol-depletion of rat platelets has the opposite effect to cholesterol-depletion of human platelets with regard to ADP induced aggregation (see Figure 7, Chapter 7). This finding emphasises the need for caution in relating results obtained in platelets from one species to another.

As mentioned previously, C_{21}-analogue enriched rat platelets underwent spontaneous aggregation on stirring in the aggregometer. The pattern of aggregation was quite different from platelets with a normal or altered cholesterol content. Figure 8.1 shows aggregation traces obtained from PRP-liposome samples containing normal platelets (trace A) cholesterol-rich platelets (trace B) C_{21}-analogue enriched platelets (trace C) and C_{24}-analogue enriched platelets (trace D). It can be seen that trace B shows an irreversible aggregation pattern whereas trace A was reversible, showing the enhanced sensitivity of cholesterol-enriched platelets. The traces of analogue enriched platelets initially had a lower optical density, indicated by the position of the recorder pen further to the right. This suggested that the platelets were either smaller and/or fewer in number, and it can be seen that aggregation was much slower than control platelets, and irreversible. C_{24}-analogue enriched platelets required prolonged centrifugation for sedimentation and were similar to C_{21}-analogue enriched platelets in this respect also.

Incorporation of C_{26}-analogue enrichment of rat platelets occurred to a lesser extent than either the C_{21}- or C_{24}-analogue (Table 8.6).

Figure 8.1. Aggregation of rat platelets after 3 hrs incubation with liposomes to alter platelet sterol content

Trace A shows reversible aggregation of control (cholesterol-normal) platelets induced by 2.4 μg/ml collagen.

Trace B shows irreversible aggregation of cholesterol-enriched platelets induced by 2.4 μg/ml collagen.

Trace C shows the spontaneous, slow, irreversible aggregation of C_{21}-analogue enriched platelets.

Trace D shows the spontaneous, slow, irreversible aggregation of C_{24}-analogue enriched platelets.

Figure 8.1.

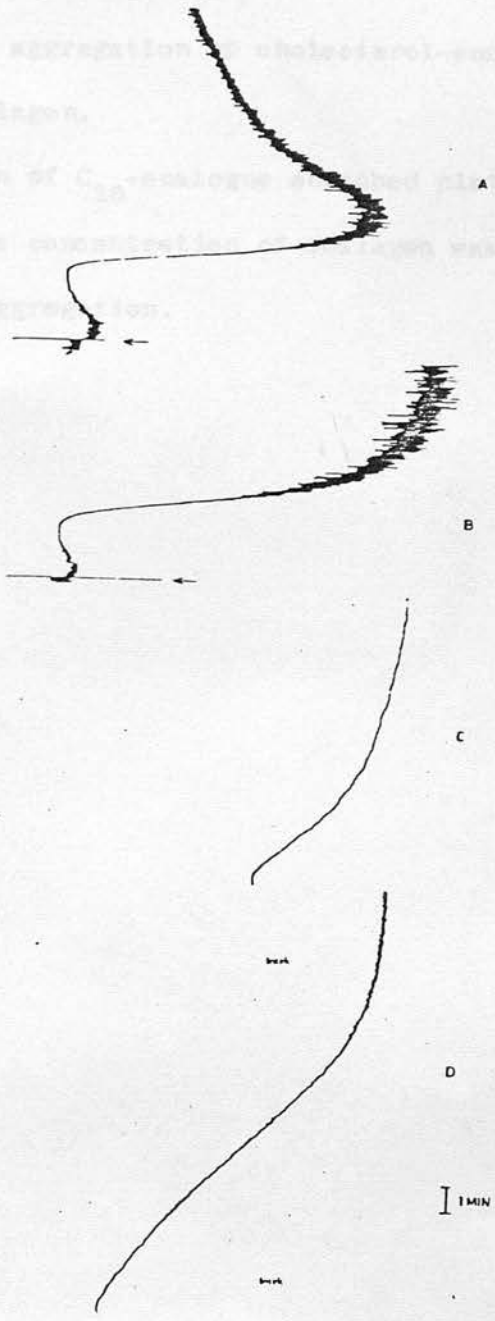


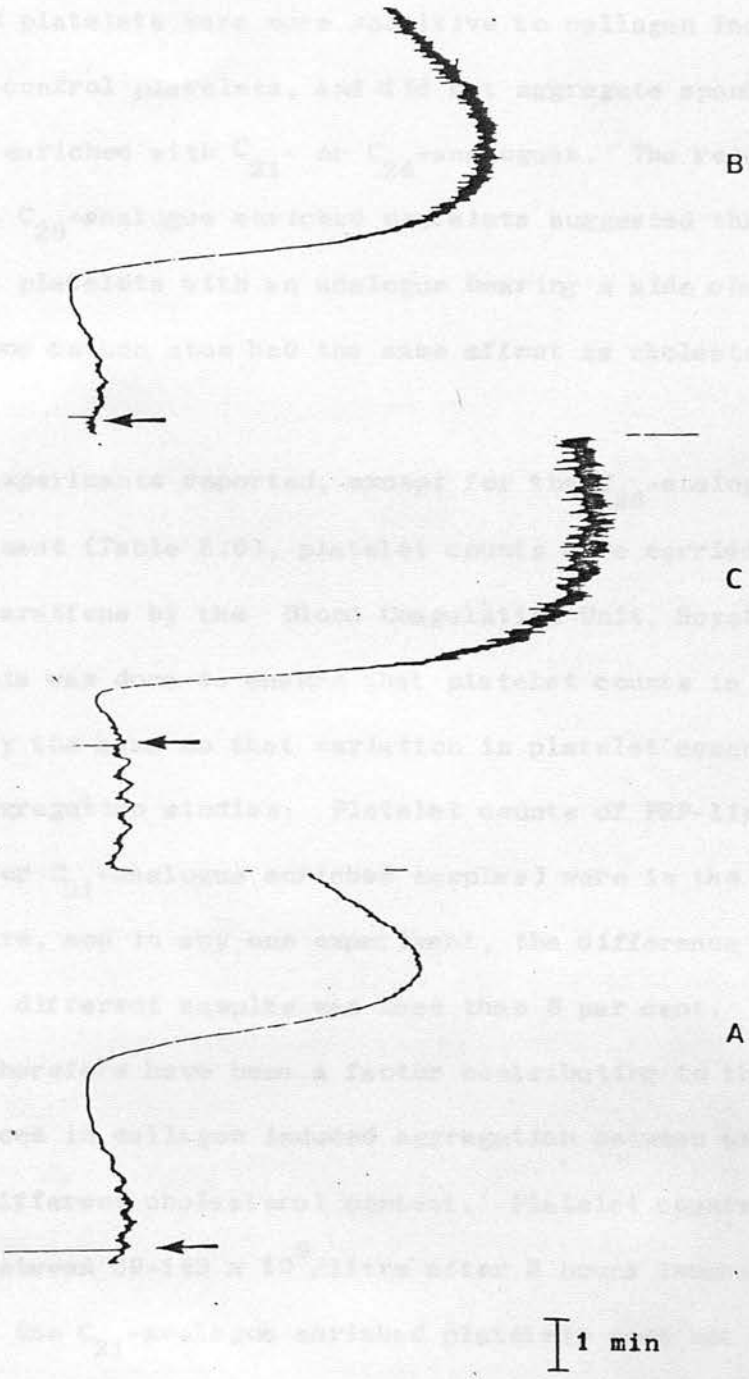
Figure 8.2. Aggregation of rat platelets after 6 hrs incubation with liposomes to alter platelet sterol content

Trace A shows reversible aggregation of control (cholesterol-normal) platelets induced by 2.0 μg/ml collagen.

Trace B shows reversible aggregation of cholesterol-enriched platelets induced by 2.0 μg/ml collagen.

Trace C shows aggregation of C_{26}-analogue enriched platelets induced by 2.0 μg/ml collagen. This concentration of collagen was almost sufficient to induce irreversible aggregation.

Figure 8.2.



C_{26}-analogue enriched rat platelets did not show similar characteristics to platelets enriched with the other analogues, but were instead very similar to cholesterol-enriched platelets. Figure 8.2 shows aggregation traces of normal platelets (trace A), cholesterol-enriched platelets (trace B) and C_{26}-analogue enriched platelets (trace C). The C_{26}-analogue enriched platelets were more sensitive to collagen induced aggregation than control platelets, and did not aggregate spontaneously as did platelets enriched with C_{21}- or C_{24}-analogues. The results obtained with the C_{26}-analogue enriched platelets suggested that enrichment of rat platelets with an analogue bearing a side chain reduced by only one carbon atom had the same effect as cholesterol enrichment.

In all the experiments reported, except for the C_{26}-analogue enrichment experiment (Table 8.6), platelet counts were carried out on PRP-liposome preparations by the Blood Coagulation Unit, Royal Infirmary of Edinburgh. This was done to ensure that platelet counts in samples were approximately the same so that variation in platelet count would not affect the aggregation studies. Platelet counts of PRP-liposome samples (except for C_{21}-analogue enriched samples) were in the range 378-542 x 10^9/litre, and in any one experiment, the difference in count between the different samples was less than 5 per cent. Platelet count would not therefore have been a factor contributing to the observed differences in collagen induced aggregation between samples of platelets of different cholesterol content. Platelet counts of C_{21}-analogue ranged between 59-143 x 10^9/litre after 3 hours incubation. It is likely that the C_{21}-analogue enriched platelets were not counted by the Coulter counter because they were too small to be detected as they passed through the 50 μ aperture. The prolonged centrifugation required to sediment C_{21}-analogue enriched platelets, and the lower

optical density of PRP-liposome samples supports the theory that these platelets have disintegrated or become reduced in size.

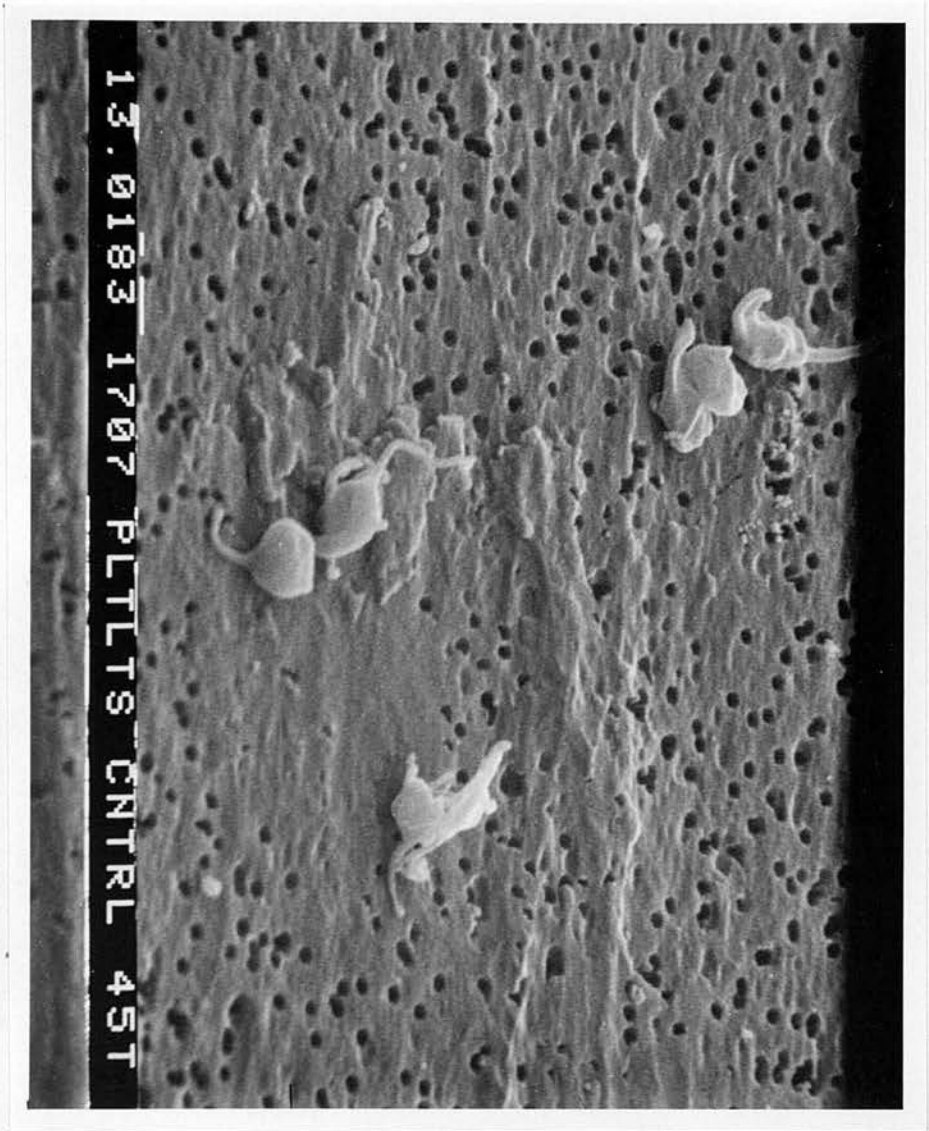
In order to investigate this possibility and to study platelets from all the incubation systems more carefully, scanning electron microscopy was carried out on platelets after incubation.

8.5 Scanning Electron Microscopy of Rat Platelets

An experiment was carried out to examine platelets after incubation of rat PRP samples with liposome suspensions. After 5 hours incubation, platelets were analysed for sterol, phospholipid and protein content (Table 8.8), and samples of platelets were prepared for scanning electron microscopy. Samples of platelets were prepared for scanning electron microscopy by resuspending platelets in an equal volume of modified Tyrode's buffer, and adding five drops of this suspension to 5 ml of glutaraldehyde fixative. Samples of platelets for scanning electron microscopy were prepared from 1) a PRP sample incubated with an equal volume of modified Tyrode's buffer containing glucose (5 mM) (Plate 8.1); 2) a PRP sample incubated with C_{21}-analogue-rich liposomes (Plate 8.2); 3) a PRP sample incubated with cholesterol-poor liposomes (Plate 8.3); 4) a PRP sample incubated with cholesterol-normal liposomes (Plate 8.4); 5) a PRP sample incubated with cholesterol-rich liposomes (Plate 8.5).

Plates 8.1 and 8.3-8.5 show that these platelet samples were slightly activated either by the resuspending process or by the incubation of 5 hours. The activation is indicated by the formation of pseudopodia on the platelet surface. This activation was not due to incubation with liposomes specifically, because platelets incubated in buffer alone show a similar morphology. Plate 8.2 shows that the morphology of platelets which were enriched with the C_{21}-analogue was very different. These platelets were reduced to between $\frac{1}{4}$ and $\frac{1}{2}$ the size of normal platelets, and they had become spherical. It also appears that some of the platelets were budding off smaller platelet vesicles. This micrograph (8.2) confirmed the decreased size of C_{21}-analogue enriched platelets suspected from the different centrifugation and aggregation characteristics discussed above.

Plate 8.1. Scanning electron micrograph (S.E.M.) of control rat platelets incubated for 5 hr at 37°C. X 2500



Plates 8.1-8.5 are S.E.M.s prepared with once-resuspended platelets. X 2500

Plate 8.2. S.E.M. of rat platelets enriched with C_{21}-analogue by incubation with C_{21}-analogue rich liposomes for 5 hr at 37°C. X 2500

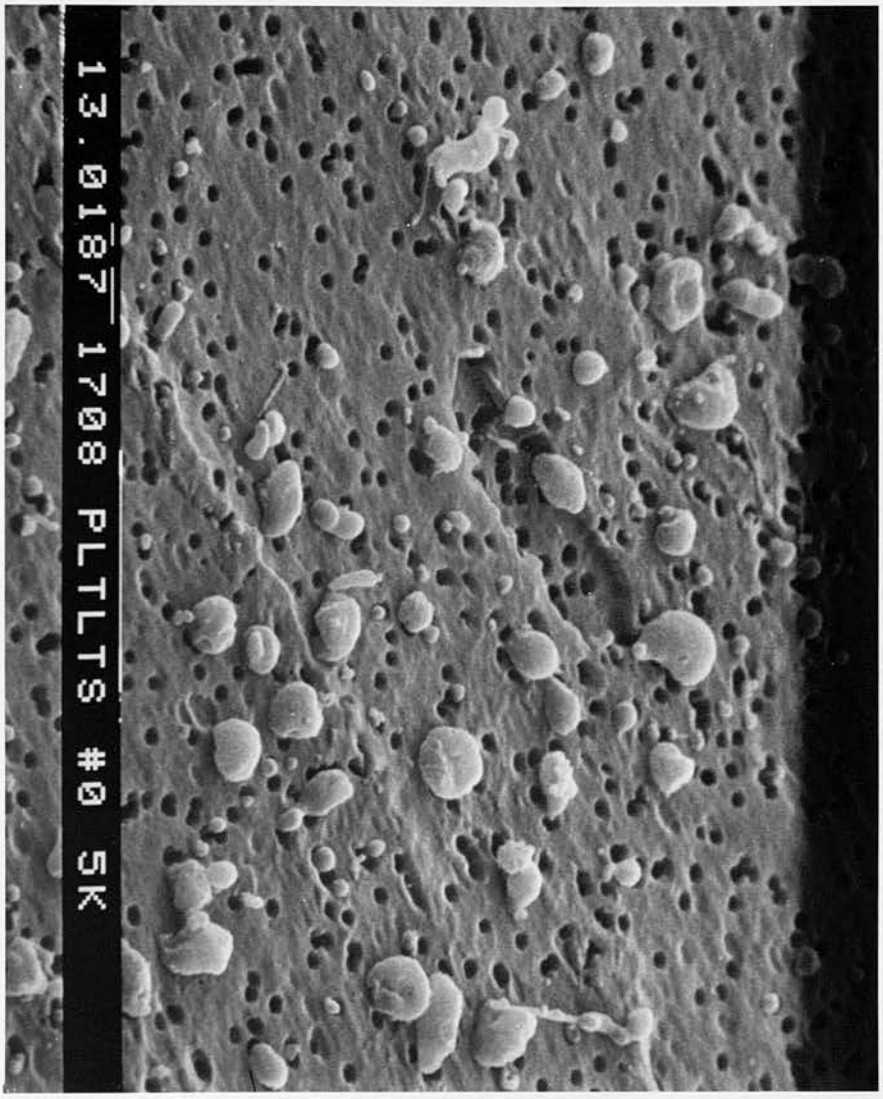


Plate 8.3. S.E.M. of rat platelets depleted of cholesterol by incubation with cholesterol-poor liposomes for 5 hr at 37°C. X 2500

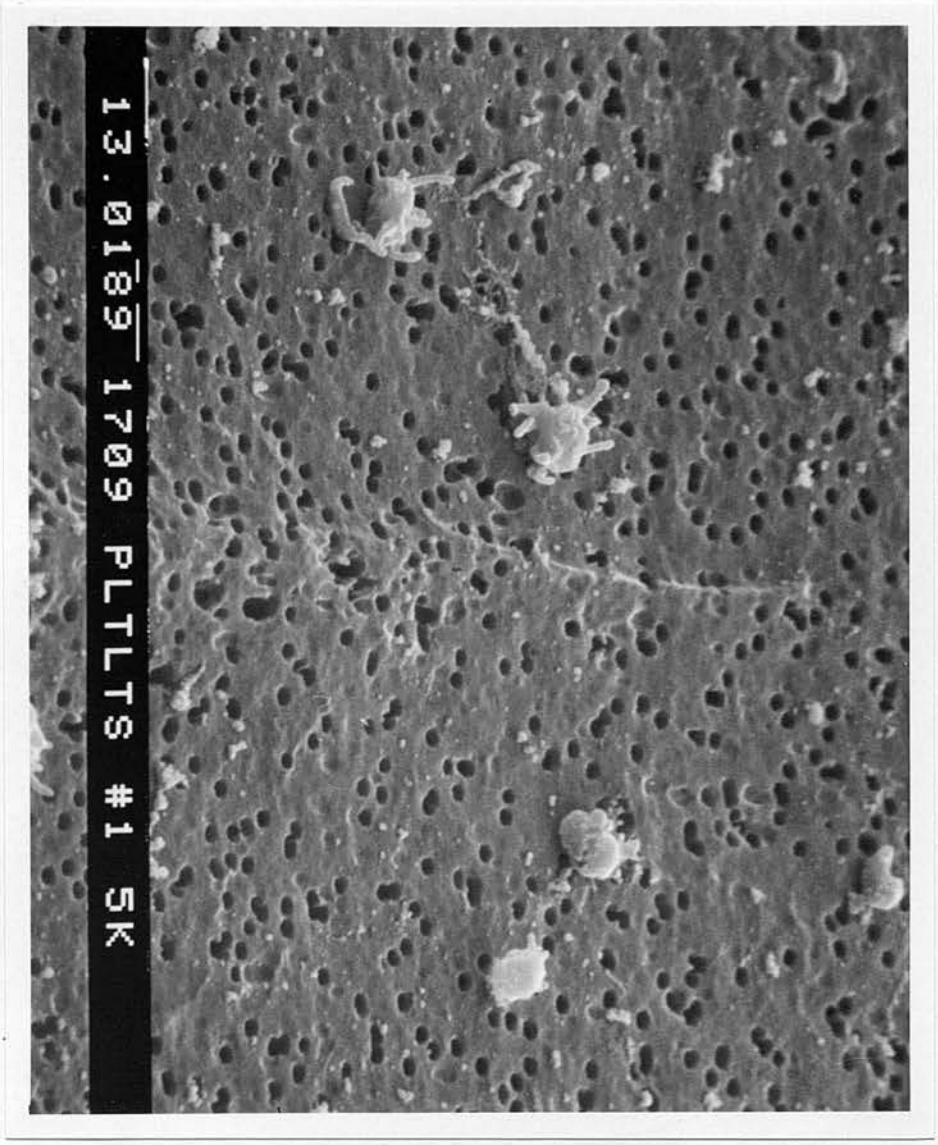


Plate 8.4. S.E.M. of rat platelets incubated with cholesterol-normal liposomes for 5 hr at 37°C. X 2500

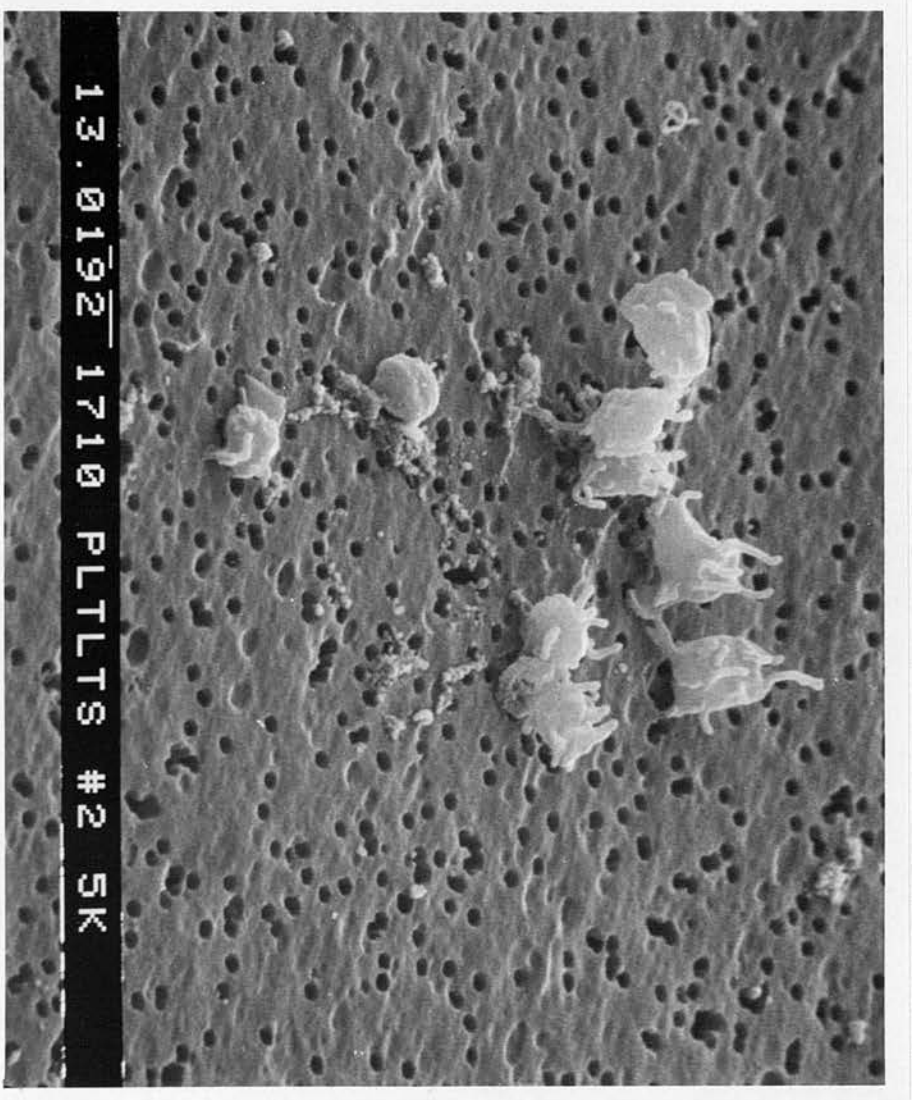


Plate 8.5. S.E.M. of rat platelets enriched with cholesterol by incubation with cholesterol-rich liposomes for 5 hr at 37°C. X 2500

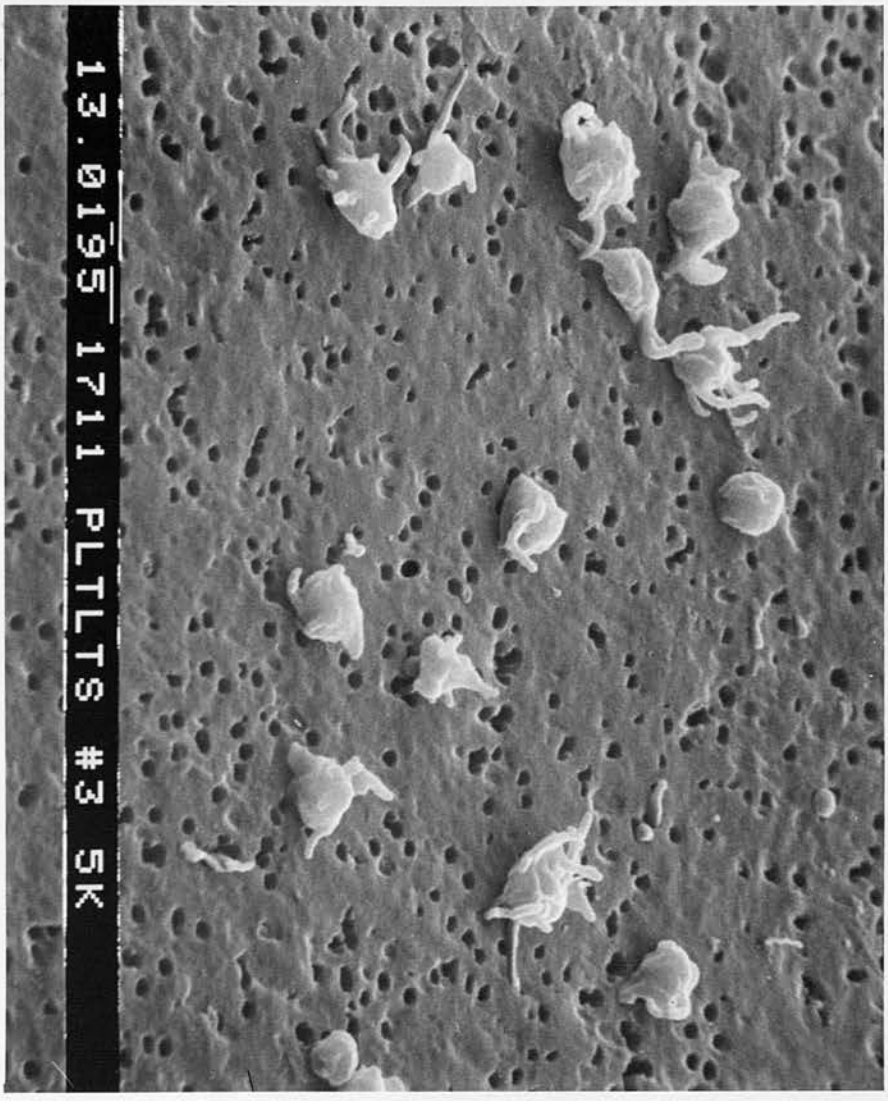
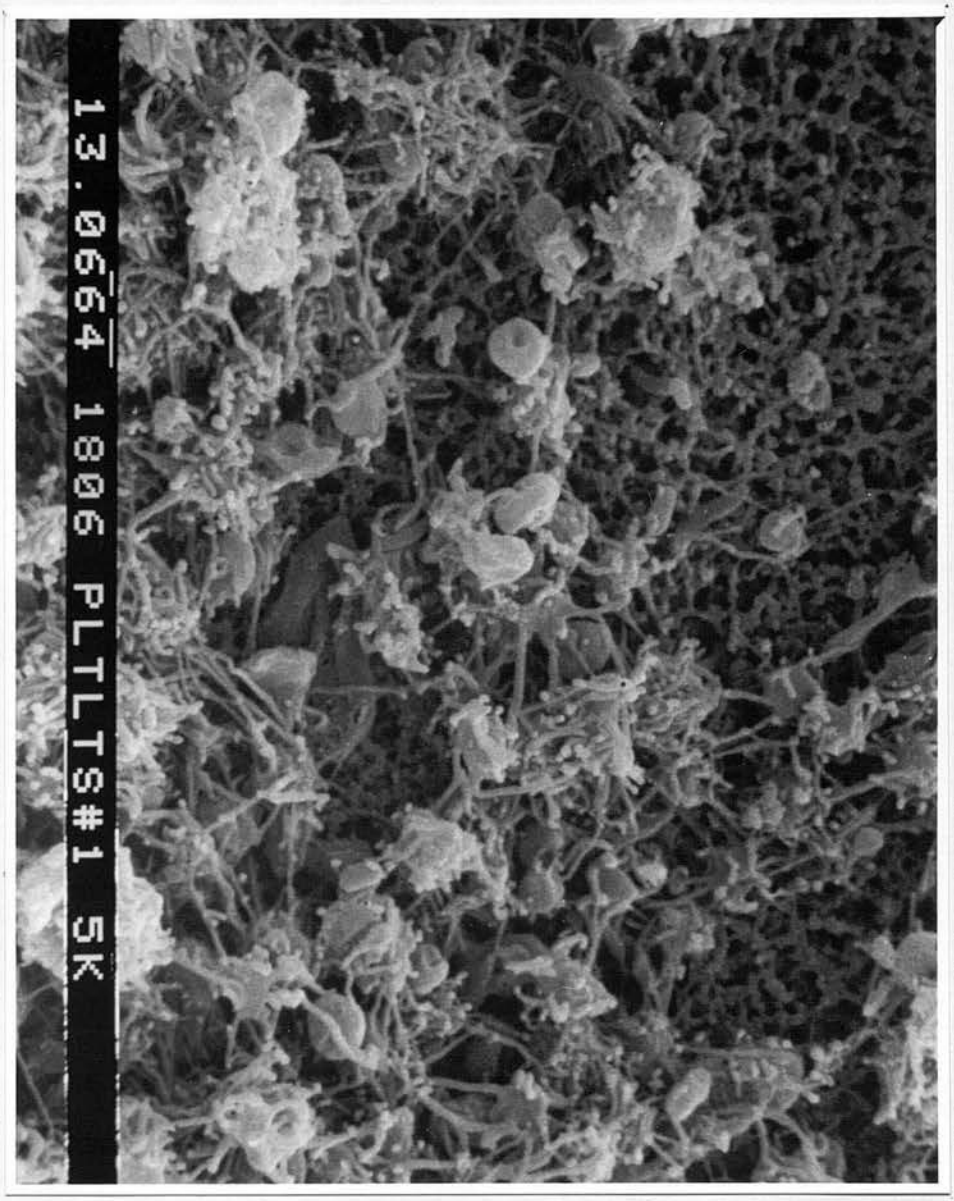
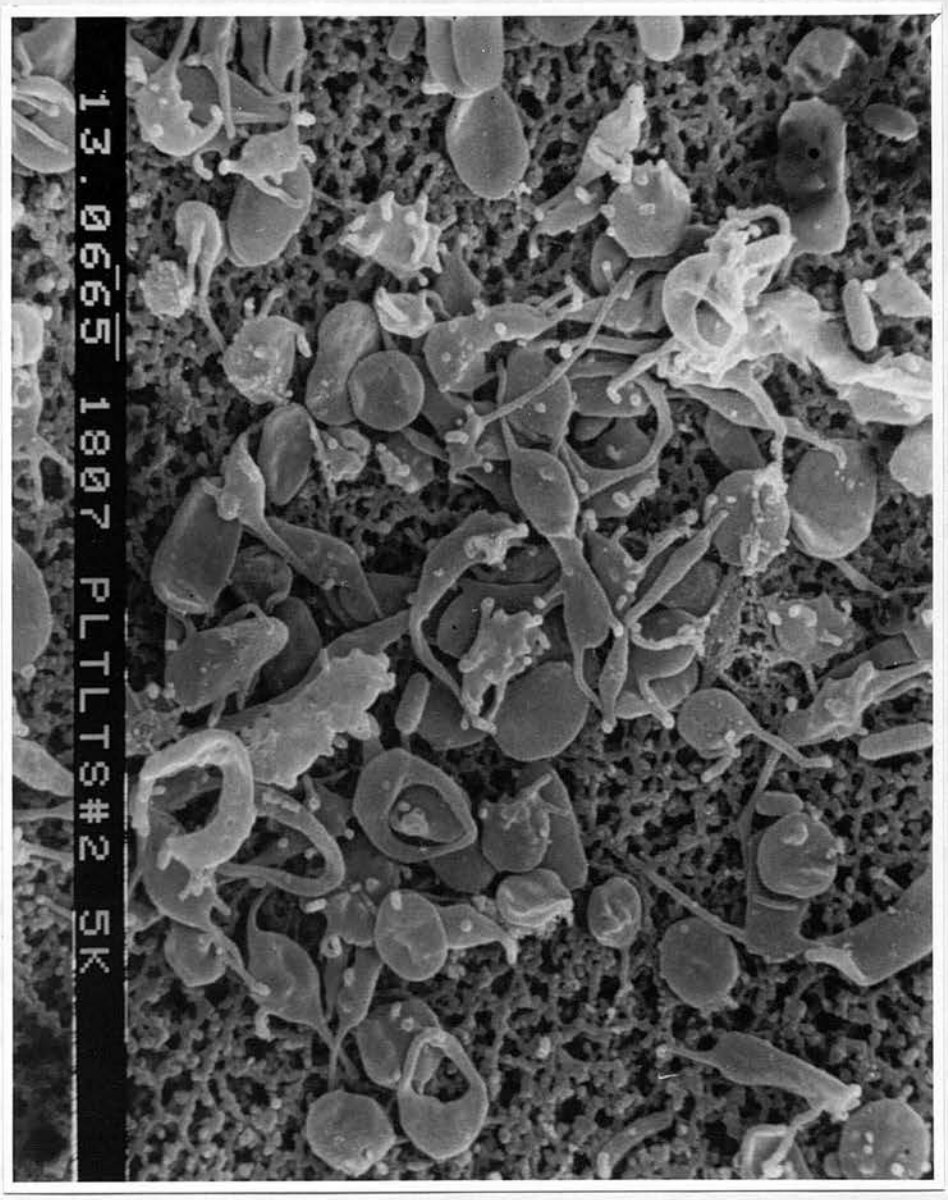


Plate 8.6. S.E.M. of PRP-liposome sample of C_{24}-analogue-enriched platelets incubated with C_{24}-analogue-rich liposomes for $3\frac{1}{2}$ hr at 37^{o}C. X 2500



Plates 8.6 and 8.7 are S.E.M.s prepared by the addition of 0.25 ml of the PRP-liposome suspension to the fixative.

Plate 8.7. S.E.M. of PRP-liposome sample of normal platelets and cholesterol-normal liposomes incubated for 3½ hr at 37°C. X 2500



In a separate experiment, scanning electron microscopy was performed on C_{24}-analogue enriched platelets and control platelets prepared after 3 hours incubation. Plate 8.6 shows C_{24}-analogue enriched platelets, and plate 8.7 shows control platelets incubated with cholesterol-normal liposomes prepared from the same experiment. In these preparations, 0.25 ml of PRP-liposome mixture was added directly to the glutaraldehyde fixative. The C_{24}-analogue enriched platelets appear smaller than control platelets, and this smaller size explains the lower optical density recorded compared to control platelets (see figure 8.1). These latter two micrographs also indicate some activation of the platelets, and since these samples were not resuspended, this suggests that rat platelets incubated for 3 hours or more are activated specifically by this incubation procedure, and do not therefore represent normal platelet physiology.

8.6 Phospholipase A_2 studies

Phospholipase A_2 assays were carried out in the experiments reported in Tables 8.1-8.5. These assays were carried out using crude membrane fractions of platelets prepared by the method described in Chapter 2. As mentioned previously, the analyses of sterol, phospholipid and protein were carried out on aliquots of each membrane preparation. Protein estimation was carried out on freshly prepared membrane fractions so that phospholipase A_2 assay systems could be set up containing the same amount of membrane protein in each.

In all the experiments carried out for assay of phospholipase A_2 except for one (see Table 8.5) the cholesterol-enriched platelet membranes consistently showed significantly higher activity than in control membranes. Cholesterol-depleted membranes (Tables 8.1-8.3) showed mean phospholipase A_2 activities which were higher than for control membranes, but this was significantly higher in only one experiment (Table 8.3). The phospholipase A_2 activity of C_{21}-analogue

enriched platelet membranes showed mean values which were generally lower than for control membranes (Tables 8.2; 8.3) but this was significantly lower in only one experiment (Table 8.3). The phospholipase A_2 activity of C_{24}-analogue enriched membranes was not significantly different from control membranes (Table 8.5).

In one experiment (Table 8.3) a sample of PRP was incubated with buffer alone. In this sample, the phospholipase A_2 activity was significantly higher than in the control sample, which indicated the possibility that phospholipase A_2 activity was affected by incubation with liposomes. However, since the cholesterol:phospholipid molar ratio in the membrane fraction of the platelets incubated in buffer alone was higher (0.620) than the control platelets incubated with cholesterol-normal liposomes (0.555), it is likely that the higher cholesterol content gave rise to the higher phospholipase A_2 activity.

8.7 Discussion

The analysis and aggregation results presented in this chapter confirmed that cholesterol enrichment of rat platelets in PRP-liposome incubation systems gave rise to enhanced sensitivity to collagen induced aggregation in these platelets compared to control platelets. These results agree with those presented in Chapter 5 which also show enhanced sensitivity to collagen of cholesterol-rich PRP-liposome samples. Enrichment of rat platelets with the C_{21}-analogue gave rise to deleterious alterations in these platelets. The aggregation pattern of these cells became very different from the normal pattern and scanning electron microscopy confirmed that gross structural alterations of these platelets had taken place. Platelets enriched with the C_{24}-analogue showed a similar aggregation pattern to C_{21}-analogue enriched platelets (Figure 8.1). It is clear from the results for aggregation obtained with platelets enriched with these two analogues, that cholesterol

is of the utmost importance for normal platelet function. These results suggest that for the normal function of platelets in haemostasis to be maintained, platelet cholesterol cannot be replaced by analogues with side chains shorter by 3 or more carbon atoms. The results obtained with C_{26}-enriched platelets (Figure 8.2) in which the normal aggregation pattern was maintained indicated that these rat platelets could perhaps tolerate partial replacement of membrane cholesterol with the C_{26}-analogue without the adverse effects observed with C_{21}- and C_{24}-analogue enrichment. A further investigation is required to test whether platelets containing a mixture of cholesterol and C_{26}-analogue with a normal total sterol:phospholipid molar ratio showed similar sensitivities to collagen induced aggregation as normal platelets. This would determine more precisely whether platelet cholesterol could be replaced with the C_{26}-analogue without affecting platelet function.

It has also been shown by the results presented in this chapter that cholesterol-enriched rat platelet membrane fractions had significantly enhanced phospholipase A_2 activity compared to control platelet membrane fractions. In Chapter 5, once resuspended rat platelet samples did not show any difference in phospholipase A_2 activities between control and cholesterol-enriched platelets. It is possible that the substrate used for the assay (exogenous radiolabelled phospholipid) was not freely available to the enzyme for hydrolysis. The delivery of substrate to the enzyme may have been a rate limiting factor, thus masking any difference in phospholipase A_2 activities between the two types of platelets. In the study presented in this chapter, sonication of membrane fractions most probably revealed the enzyme to make it more available to the substrate.

In Chapter 6 of this thesis, cholesterol-enriched platelets were obtained from rabbits fed a cholesterol-rich diet. These platelets were

compared with platelets obtained from normal rabbits. It was shown in once resuspended rabbit platelets, that cholesterol-enriched platelets had a higher phospholipase A_2 activity compared to control platelets, when this activity was assayed after stimulation with collagen under specific conditions. The results reported from the rabbit study in Chapter 6, and the results presented in this chapter, strongly suggest that the enhanced sensitivity of cholesterol-enriched platelets to collagen induced aggregation may in part be mediated through an increased activity of phospholipase A_2 catalysed hydrolysis of membrane phospholipids. The mode of action by which cholesterol enrichment may make this enzyme hyperactive remains unclear, but it is possible that in cholesterol-enriched platelet membranes the phospholipid substrate is made more readily available to phospholipase A_2.

Chapter 9

General Discussion

Extensive research has been carried out over the last two decades to investigate the factors responsible for inducing thrombosis and atherosclerosis. Hypercholesterolaemia is generally considered to be a possible factor, and many projects have been conducted to investigate this in human subjects and in animal models. Platelets are found in atherosclerotic plaques and thrombi (Haft, 1979) and it is generally accepted that platelets are involved in the development of these disorders.

Human subjects with type lla hyperbetalipoproteinaemia have a high risk of severe early age atherosclerosis (Lees et al., 1973). Shattil et al. (1975) showed that when normal human platelets were enriched with cholesterol *in vitro* they became more sensitive to the aggregating agents ADP and adrenaline. Shattil et al. (1977) showed that platelets from type lla subjects had a raised cholesterol content and were hypersensitive to aggregating agents. Stuart et al. (1980a) used the same *in vitro* cholesterol enrichment and depletion technique as used by Shattil et al. They showed that cholesterol-enriched human platelets synthesised greater quantities of arachidonic acid metabolites in response to thrombin stimulation compared to platelets depleted of cholesterol. Worner and Patscheke (1980) also used this *in vitro* technique, and demonstrated enhanced arachidonic acid metabolism in cholesterol-enriched human platelets compared to control human platelets.

The evidence discussed above suggested the possibility that cholesterol enrichment of platelets may have given rise to hypersensitivity in part through enhanced arachidonic acid metabolism. Phospholipase A_2 is considered to be an important enzyme responsible for the release of arachidonic acid from platelet phospholipids (see for example Blackwell et al., 1977). More recently a different pathway for the release of arachidonic acid has been identified. A phospholipase C enzyme which specifically cleaves phosphatidylinositol at the sn-3 position to

produce a diacylglycerol has been well characterised (Billah et al., 1980). This enzyme is present in the cytosol of the platelet whereas the phospholipase A_2 is membrane bound. The diacylglycerol produced by phospholipase C activity is then available for hydrolysis by diacyl glycerol lipase to release arachidonic acid (Bell et al., 1979). The proportion of free arachidonic acid generated by these two pathways to the total pool available for further metabolism in activated platelets is the subject of much current research and will be discussed later.

The aim of the present project was to investigate the effect of alteration of the cholesterol content of platelet membranes on platelet function. Phospholipase A_2 may be an important rate limiting step in the metabolism of arachidonic acid to the pro-aggregating agents PGH_2 and thromboxane A_2, therefore experiments were carried out to investigate the influence of membrane cholesterol content on this membrane bound enzyme. Two principal approaches were undertaken in this project to alter the cholesterol content of platelets. These were 1) *in vitro* alteration essentially by the method of Shattil et al. (1975) as described in Chapter 3 and 2) *in vivo* enrichment of platelets by feeding rabbits a diet supplemented with cholesterol (Chapter 8).

These two methods are referred to as the "in vitro" and "in vivo" methods of alteration of platelet cholesterol content. Rat platelets were enriched with cholesterol using the *in vitro* method (Chapter 3). After 3 hours incubation with cholesterol-rich liposomes, rat platelets were enriched with cholesterol by between 10 to 40 per cent which resulted in enhanced sensitivity to collagen induced aggregation (Chapters 5 and 8). The same technique was used to enrich and deplete human platelets of cholesterol (Chapter 7). In human platelets, cholesterol-depletion gave rise to reduced sensitivity, and cholesterol-enrichment gave rise to enhanced sensitivity to ADP induced aggregation.

This is in agreement with the results of Shattil et al. (1975). Rat platelets enriched with cholesterol consistently showed greater sensitivity to collagen than control rat platelets. However, in any one experiment few aggregation tests were performed. In some cases only one sample was tested. As was discussed in Chapter 5, samples of rat PRP obtained from different groups of animals demonstrated widely different sensitivities to collagen. It has not been possible therefore to make a statistical analysis on the rat platelet experiments in order to test the significance of differences between control and cholesterol enriched platelets with respect to aggregation. In future studies therefore it would be possibly more suitable to determine platelet sensitivity by using threshold concentrations of aggregation agents. This would be expected to induce aggregation in the more sensitive platelet sample, whereas no response would be elicited in the less sensitive sample.

In addition to cholesterol enrichment of rat platelets, three experiments are reported where rat platelets were depleted of cholesterol (Chapter 8). In two experiments, cholesterol depletion resulted in enhanced sensitivity to collagen induced aggregation, whereas in one experiment this sensitivity was reduced when compared to control rat platelets. Interpretation and explanation of these findings are severely restricted due to the low number of experiments performed. However, these results contrast markedly when compared with results obtained with human platelets. Cholesterol depletion of human platelets leads consistently to decreased sensitivity to aggregation as shown in the present studies (Chapter 7) and in the current literature as previously discussed. These 'different' results of rat platelets and human platelets serves to highlight the difficulty of interpreting data obtained from different species along a common biochemical mechanism.

It is very evident that platelets from different species have important differences in their biochemistry. Much of the work presented in this thesis has been carried out on rat platelets and some on rabbit platelets. This therefore severely limits interpretation of data in the human context.

Scanning electron microscope studies of rat platelets incubated with liposomes for three and five hours have been carried out (Chapter 8). This is discussed in more detail below, but as has been pointed out in Chapter 8, this incubation procedure may cause activation of platelets. This was demonstrated by the micrographs which showed that platelets had developed small pseudopodia. The possibility that the incubation procedure may activate platelets has been demonstrated and cannot be excluded. It is evident however that the liposomes are not responsible for this activation since platelets incubated in buffer alone showed the same characteristics.

In the experiments discussed in Chapter 5, phospholipase A_2 activity was assayed in once resuspended rat platelets. No difference in activity of this enzyme was detected between cholesterol-enriched and control (cholesterol-normal) platelets. As has already been mentioned, cholesterol-enriched rat platelets showed enhanced sensitivity to aggregating agents. It was observed that cholesterol-enriched platelets that had been resuspended once showed a very much reduced sensitivity to collagen induced aggregation compared to control platelets. It is possible that the resuspending process activated the cholesterol-enriched platelets to a greater extent than control platelets, giving rise to the reduced sensitivity to collagen and reduced phospholipase A_2 activity. This may explain why the phospholipase A_2 activities in cholesterol-enriched and control platelets were not different as measured in once resuspended platelet samples.

The alternative appraoch to enrich platelets with cholesterol, referred to here as the *in vivo* method was carried out by feeding a group of rabbits a diet supplemented with cholesterol (Chapter 6). This dietary regimen raised the serum cholesterol level, and resulted in an increase in the platelet unesterified cholesterol:phospholipid molar ratio compared to platelets from rabbits fed a normal diet. The cholesterol-enriched platelets from the cholesterol fed group showed a significantly more active arachidonic acid metabolic pathway. The phospholipase A_2 activity of once resuspended cholesterol-enriched rabbit platelets was enhanced compared to once resuspended platelets from rabbits fed a normal diet. The enhanced activity was only detected under very precise conditions (see Chapter 6 for details). Phospholipase A_2 activity was significantly increased in cholesterol enriched samples when resuspended platelets were stimulated with collagen (2 µg/ml) in the presence of 0.2 mM Ca^{2+}. In unstimulated samples, average phospholipase A_2 activity was increased in cholesterol-enriched compared with control platelets, but this was not significant at the $p = 0.05$ level. When the experiment was repeated using twice the concentration of Ca^{2+} and twice the final concentration of collagen, phospholipase A_2 activity was stimulated equally in cholesterol-enriched and control platelet samples.

It was recently reported by Kawaguchi et al. (1981) that the phospholipase A_2 activity in platelets isolated from normal rabbits was not different from the activity in platelets isolated from rabbits fed a cholesterol-enriched diet. As was discussed in Chapter 6, the conditions used by this group to assay phospholipase A_2 probably stimulated the enzyme superoptimally so as to effectively hide any subtle difference. It would appear from the results discussed above and in Chapter 6 that very precise conditions are required to assay phospholipase A_2 activity and to show differences in phospholipase A_2 activity between normal and cholesterol-enriched platelets isolated from rabbits.

As discussed above, rat platelets enriched with cholesterol by the in vitro technique and resuspended once did not show any difference in phospholipase A_2 activity compared to control platelets treated in the same way. A possible reason for this may have been that the rate of delivery of substrate to the enzyme was limited or restricted. Further experiments were carried out to test the viability of assaying phospholipase A_2 activity in crude platelet membrane preparations (see Chapter 4). It was shown that rat platelet membranes provided a good source of phospholipase A_2. In view of this, experiments were carried out to assay phospholipase A_2 activity in crude rat platelet membrane fractions prepared from platelets enriched with cholesterol by the in vitro technique (Chapter 8). It was consistently shown that cholesterol-enriched platelet membranes had a significantly higher phospholipase A_2 activity than control platelet membranes. Phospholipase A_2 assays were also carried out on crude membrane fractions prepared from cholesterol depleted rat platelets. In two experiments there was no significant difference compared to control samples, whereas in one experiment there was a significant increase in phospholipase A_2 activity. As discussed previously, interpretation of few results is risky, but the mean levels of phospholipase A_2 activity in all three experiments were higher than the control samples. This suggests that the normal cholesterol: phospholipid molar ratio may maintain a degree of inhibition of phospholipase A_2 activity in rat platelets, but in further experiments larger numbers of samples would be required. In the three experiments reported here, only four samples of crude membrane fraction were assayed for phospholipase A_2 activity which reduces the reliability of statistical analysis.

A trial was also carried out to study platelets isolated from human hypercholesterolaemic subjects. This study was of interest because

Shattil et al. (1977) had shown that platelets from type 11a hyperbeta-lipoproteinaemic subjects were hypersensitive to aggregating agents compared to normal human platelets. In order to study phospholipase A_2 activity, crude human platelet membranes were prepared by the method described for rat platelets. No difference in phospholipase A_2 activity was detected in platelet membranes prepared from the hypercholesterolaemic and control groups of subjects (Chapter 7). Phospholipase A_2 activities in human platelet membranes were very low, which may explain why no differences were observed.

The investigations carried out in this project showed that cholesterol-enrichment of rat and rabbit platelets resulted in enhanced phospholipase A_2 activity. The mechanism by which this effect occurred remains unclear, but there are a number of possibilities:

1. Cholesterol may exert a direct effect on the phospholipase A_2 enzyme.

2. Cholesterol may exert an effect on phospholipids in the membrane which in turn affect the enzyme or

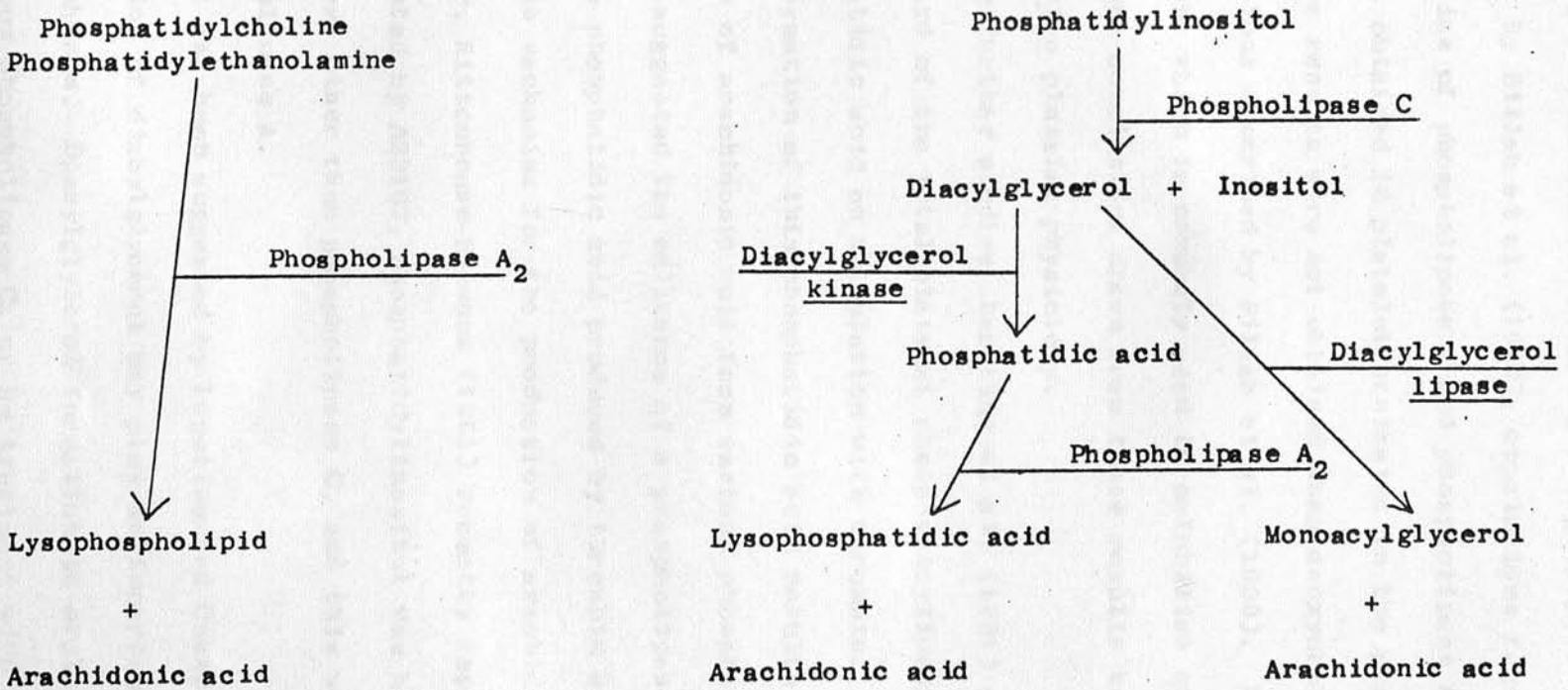
3. cholesterol may have an effect on phospholipids which affects the properties of the substrate phospholipid.

The technique developed in the present study could be used for further investigations. Of particular interest would be the study of phospholipase A_2 activity in cholesterol-enriched platelets in the presence of anti-lipaemic drugs. Colman et al. (1976) showed that

halofenate reduced the hypersensitivity to aggregating agents of platelets isolated from type lla hyperbetalipoproteinaemic subjects. Carvalho et al. (1974b) showed similar results with clofibrate. Cholesterol-enriched platelets could be assayed for phospholipase A_2 activity in the presence of these drugs. This would show whether the anti-platelet effect of these drugs was due to a reduction of arachidonic acid metabolism by phospholipase A_2 inhibition.

Recently much research has been carried out to determine the source of arachidonic acid which is liberated from membrane phospholipids when platelets are stimulated. Also, the pathways of release of arachidonic acid from membrane phospholipids have been investigated. It was generally accepted that phospholipase A_2 was the enzyme responsible for hydrolysis of arachidonic acid from platelet membrane phospholipids (Blackwell et al., 1977; Smith et al., 1976). Very recently, it has become clear that phospholipase A_2 activity is not the only pathway for arachidonic acid release. A phospholipase C has been identified which cleaves specifically phosphatidylinositol at the phosphate bond to produce the corresponding diacylglycerol (Rittenhouse-Simmons, 1979). The diacylglycerol is then available for further metabolism by two enzymes (see Figure 9.1). Diacylglycerol kinase, identified by Call and Rubert (1973) forms phosphatidic acid from the diacylglycerol. This pathway has been investigated by several researchers (Billah et al., 1979; Lapetina and Cuatrecasus, 1979; Broekman et al., 1980). It has been suggested by Bell et al. (1979) that a diacylglycerol lipase exists which hydrolyses the arachidonic acid from the sn-2 position of the glycerol moiety. It was shown by Broekman et al. (1979) that it was only phosphatidylinositol which decreased in mass in platelets on stimulation with thrombin which suggested that phospholipase A_2 activity was less important in providing free arachidonic acid than the phospholipase C/diacylglycerol lipase pathway.

Figure 9.1. Pathways of arachidonic acid release from platelet membrane phospholipids



Billah et al. (1980) suggested that when platelets are stimulated with thrombin, the cytosolic phospholipase C is firstly stimulated to produce diacylglycerol which is converted to phosphatidic acid by the kinase. This phosphatidic acid may then act as a calcium ionophore which is responsible for inducing phospholipase A_2 activity. In the studies by Billah et al. (1980), conclusions regarding the different properties of phospholipase C and phospholipase A_2 were based on the results obtained in platelet sonicates in the presence of deoxycholate. The same results were not obtained when deoxycholate was excluded from incubations described by Billah et al. (1980). Deoxycholate is a detergent which is commonly used to solubilise or disperse membranes, therefore conclusions drawn from these results may not be relevant to *in vivo* platelet physiology.

In further studies, Lapetina et al. (1981) provided evidence that one third of the total platelet phosphatidylinositol was converted to phosphatidic acid on stimulation with thrombin. These workers suggested that formation of this phosphatidic acid results in the subsequent release of arachidonic acid from various phospholipids. Billah et al. (1981) suggested the existence of a phospholipase A_2 activity specific for the phosphatidic acid produced by thrombin stimulation as being a possible mechanism for the production of arachidonic acid in platelets. However, Rittenhouse-Simmons (1981) recently reported that in platelets stimulated by A23187, phosphatidylinositol was hydrolysed primarily by an enzyme other than phospholipase C, and this may have been a phospholipase A.

It has been suggested by Lapetina and Cuatrecasus (1979) that the formation of diacylglycerol may play an important role in platelet shape change. Diacylglycerol formation in erythrocytes induced by exogenous phospholipase C, or by treatment with ionophore A23187 is

thought to be, at least in part, responsible for shape change, microvesicle formation and membrane curvature (Allan et al., 1978). Further research which could be carried out of relevance to the present project would be investigations into the phospholipase C activity of cholesterol-enriched platelets. It is possible that cholesterol-enriched platelets, as well as showing enhanced phospholipase A_2 activity, have a raised phospholipase C activity giving rise to increased amounts of diacylglycerol. The result of this might be to make the platelets change shape more readily. This, together with enhanced phospholipase A_2 activity to synthesise increased quantities of PGH_2 and thromboxane A_2 could explain the observed hyperaggregability of these platelets.

As was discussed previously (Chapter 1), it has been suggested that cholesterol fitted very precisely into artificial membranes. Liposome lipid bilayers had reduced rigidity when they contained analogues of cholesterol with different side chain lengths instead of cholesterol (Suckling et al., 1979). The platelet membrane has the specialised function of changing shape, and was therefore a suitable biological membrane in which to study the precise requirement for the cholesterol side chain. Rat platelets have been enriched with three cholesterol analogues of shorter side chain length than cholesterol in the present study (Chapter 8). The in vitro method was used to enrich rat platelets with these sterols. Initial observations of platelets enriched with the C_{21}-analogue suggested that major changes of platelet structure had occurred. Spontaneous aggregation of these platelets occurred on stirring PRP-liposome samples in an aggregometer. The pattern of aggregation was abnormal as shown by the recorded traces (Chapter 8). It was also noticed that prolonged centrifugation was required to sediment these platelets. These findings suggested that the platelets had become smaller and lighter. Scanning electron microscopy confirmed this

(Chapter 8, Plates 8.1-8.7). Platelets enriched with the C_{24}-analogue showed similar characteristics to C_{21}-analogue-enriched platelets, but C_{26}-analogue-enriched platelets showed characteristics very similar to cholesterol-enriched platelets. C_{26}-analogue-enriched platelets showed enhanced sensitivity to collagen, and the pattern of aggregation was the same as the pattern for cholesterol-enriched platelets. These results show that analogues with the shorter side chains have the most disruptive effect on rat platelets.

The sterol content has been shown to be maintained precisely for the stability of red cell membranes (Lange et al., 1980). The studies carried out in the present project suggest this may also be true for platelets. Very recently, Lange et al. (1981) have provided evidence that the movement or flip-flop of cholesterol between the two leaflets of the red cell membrane bilayer is extremely fast (3 seconds). These workers suggested that a rapid redistribution of bilayer sterols could relieve the resistance of a membrane to bend and therefore facilitate and stabilise changes in shape. It is possible that this mechanism is more easily initiated in red cells and platelets that are enriched with cholesterol. The unusual characteristics which have been observed in C_{21}-analogue and C_{24}-analogue-enriched rat platelets may be due to three most likely factors.

1) Displacement of membrane cholesterol by an analogue of significantly different dimensions may disrupt interactions between membrane proteins and contractile proteins so as to cause sphere formation

2) The cholesterol flip-flop mechanism suggested by Lange et al. (1981) for red cells, if it exists in platelets, may be interfered with by cholesterol analogues

3) Enzymes which are responsible for the formation of products concerned with shape change may become activated as a result of

enrichment of the platelet with cholesterol analogue. Clearly these sterol analogues do not have the precise properties required to maintain platelet integrity.

The effects of the cholesterol analogues on platelet aggregation and of cholesterol on phospholipase A_2 activity reported in this thesis further demonstrate the importance of the sterol component in the platelet membrane. Effects can be observed at the cellular and enzyme level and it is likely that cholesterol-enrichment of platelets induces hypersensitivity to aggregating agents through several mechanisms.

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